

BIOLOGICAL INDICATOR

5 The invention relates to a biological indicator suitable for the validation of processes, including heat-based inactivation processes and inactivation procedures in general, and more specifically for the validation of procedures to inactivate transmissible spongiform encephalopathy (TSE) agents.

10 Creutzfeldt-Jakob Disease (CJD) is a relatively rare form of human neurodegenerative disorder presenting as either a familial, sporadic or iatrogenic disease at a frequency of approximately 1 case per million population. The emergence of a new variant form (vCJD) of the disease, predominantly in a younger age group, and possibly due to consumption of bovine spongiform encephalopathy (BSE)-infected meat products, has raised
15 the possibility of a large increase in the numbers of cases. These factors have important public health consequences. A large proportion of the UK population has potentially been exposed to the disease via food during the late 1980s. Whilst the number of cases to date has been relatively low (149 cases to February 2004) there remains a significant risk, from all forms of CJD, via
20 other transmission routes including surgery, transplants, transfusion or contaminated medical products. A number of these routes have been implicated in the iatrogenic spread of the disease in a clinical setting and others have been defined in animal models.

25 The agents responsible for causing all forms of CJD in humans are highly resistant to inactivation by standard methods. Validated methods for the decontamination of surgical instruments are urgently required. A variety of treatments, including chemical treatments and the use of high temperatures and pressures with wet or dry heat, have been tested but none are adequate
30 [Taylor, D.M. (1999) in: Principles and practice of disinfection, preservation and sterilisation. (Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J., Eds): pp 222-236 Blackwell Scientific Publications, Oxford; Taylor, D.M.. (2001) Contrib Microbiol. 7, 58-67; Taylor, D.M., Fernie, K, Steele, P.J., McConnell, I. and Somerville, R.A. (2002) J Gen Virol. 83, 3199-3294]. Incineration is effective,

but precludes any recovery or reuse of raw materials or equipment. The use of high concentrations of sodium hydroxide (up to 2M) or high levels of sodium hypochlorite (up to 20000ppm) have been shown to significantly reduce the levels of TSE agents, but have a deleterious effect on surgical instruments and may be harmful to the operator. A wide variety of other methods have been proposed as means of inactivating TSE agents on surgical instruments and are currently in development. These include a variety of gaseous phase sterilants including vapour phase hydrogen peroxide, ozone and ethylene oxide. Other methods have been proposed as a specific anti-TSE pre-treatment prior to routine sterilisation and these include treatment of the surgical instruments using thermostable proteases under defined conditions of pH and temperature.

Inactivation of *Bacillus stearothermophilus* spores is a method routinely used to validate the correct performance of autoclaves. Such indicators may represent a relevant indication that a bacteria or virus has been inactivated by the process, with the process usually validated as reducing the level of infectious agent by an order of 10^6 . However, the uniquely stable properties of the TSE agents mean that a much more robust indicator is required to provide a relevant indication of the performance of processes to inactivate such agents.

Other biological indicators, based on thermostable spores or enzymatic preparations are well known to those familiar with the art. All have the drawback that they are unable to validate inactivation of an infectious agent in excess of a 10^6 reduction in activity.

Inactivation of TSE agents is also a significant issue for the disposal of Bovine Spongiform Encephalopathy (BSE)-infectious materials and in the preparation of raw materials of animal origin. There is now a good body of scientific evidence that the emergence and spread of BSE was via changes in rendering practice with highly infectious neuronal tissue being fed back to cattle via meat-and-bone-meal supplements. There is also good evidence that BSE was the cause of vCJD, almost certainly as the result of eating

contaminated beef products. For this reason any cattle that die of BSE, together with spinal cord and brain tissue from all cattle, are currently removed from the food chain and disposed of by an alternative route. This has the result that enormous amounts of animal waste is currently being
5 accumulated or disposed of by incineration. The treatment of such material with thermostable proteases is one possible solution. Again there is a requirement for a validation procedure to ensure that any infectious material is destroyed in an appropriate process.

10 It is an object of the present invention to provide an alternative and/or improved biological indicator and uses thereof.

Accordingly, in a first aspect of the invention, there is provided a biological process indicator for validating a treatment process for reducing the amount or
15 activity of a biological agent in a sample, comprising a kinase. In a preferred embodiment, the indicator further comprises a solid support, wherein the kinase is immobilised in or immobilised on said solid support. In a particularly preferred embodiment, the kinase is a thermostable kinase.

20 In a use of the invention, a biological indicator is included in a sample that is being subjected to a treatment that is intended to reduce its content of a potential contaminant, especially an infectious agent. It is known from previous tests that the reduction in activity of the indicator kinase by the treatment can be correlated with reduction in amount or activity of the
25 contaminant. To determine whether the amount/activity of the contaminant has been reduced below an acceptable level, the activity of the indicator kinase is measured before and after, or during, the treatment. When a level of activity is reached that is known to correlate with an acceptable reduction in the contaminant, the treatment is then regarded as validated. If the
30 contaminant is an infectious agent, then the sample may be regarded as sterile.

In a particular use of the invention, thermostable kinase is the reporter in a method of indicating the possible presence of an agent (e.g. an infectious

agent) following a cleaning or inactivation procedure. First, a sample containing thermostable adenylate, acetate or pyruvate kinase is exposed to a cleaning/inactivation procedure (e.g. one or more of a selected temperature, pH or protease concentration). The next step is to remove any contaminating enzymatic activity by heat treatment, e.g., at from 60 to 80°C for at least 10 minutes (i.e. under conditions that do not significantly affect the thermostable kinase). The thermostable kinase is then reacted at a temperature of between 30°C and 70°C with a substrate (e.g. ADP) to allow the generation of ATP. The formation of ATP can be measured by bioluminescent detection using luciferin / luciferase and a suitable luminometer at 20-30°C for 10 minutes to 1 hour. The reading from the luminometer gives a reading of the residual kinase activity, i.e. the activity of the kinase following exposure to the cleaning/inactivation treatment. Based on data that have been previously derived from separate experiments, the method is completed by correlating the residual kinase activity with the possible presence of an infectious agent within the treated sample.

In one embodiment, contaminating enzymatic activity or ATP in a sample may be removed by an initial treatment step (e.g. a selected temperature, pH or protease concentration), prior to addition of the indicator.

Kinase enzymes have been found to be capable of generating a signal that is detectable over an extremely wide range. Generally, the kinase is detected using a substrate comprising ADP which is converted to ATP, itself used to generate light, eg. using luciferin/luciferase, detected using a luminometer. The wide range makes the indicator particularly suitable for validation as the kinase remains detectable even after many logs reduction in amount/activity. For sterility, most national institutes regard a 6 log reduction in the amount or activity of a biological agent as required before sterility can be validated. The kinases of the invention offer the potential of validating reduction in the amount or activity of agents well beyond 6 logs, to 8 logs and more, thus increasing the scope of monitoring offered at present.

In preferred embodiments of the invention, the kinase is thermostable, and is hence suitable for use in validation of processes carried out at high temperature. Thermostable kinases are also found to be resistant to other extreme environments, and are for example often found resistant to extremes of pH and resistant to exposure to proteolytic enzymes. So the kinases of the invention can be used for monitoring treatments of biological agents that employ one or a combination or all of high pH, high temperature and proteases.

By thermostable is meant that at least 95% of the activity of the kinase is retained after exposure to 70 degrees C for 30 minutes. Preferred enzymes of the invention are very thermostable and will retain at least 95% activity after heating to 80 degrees C for 10 minutes. The kinases from mesophilic organisms and even a variety of thermophilic organisms, such as *Bacillus stearothermophilus* (used widely as a biological indicator) do not meet these criteria, but may nevertheless be suitable as indicators for treatments carried out at lower temperatures.

The kinases used in particular embodiments of the invention are adenylate kinase, acetate kinase and pyruvate kinase, or combinations thereof. Further, the adenylate, acetate and pyruvate kinase enzymes may be obtained from *Pyrococcus furiosus*, *P.abyssi*, *P.horikoshii*, *P.woesii*, *Sulfolobus solfataricus*, *S.acidocaldarius*, *S.shibatae*, *Rhodothermus marinus*, *Thermococcus litoralis*, *Thermatoga maritima*, *Thermatoga neapolitana* and *Methanococcus spp.* Adenylate kinase is especially preferred and has been used in examples of the invention set out in detail below. The kinases catalyse formation of ATP from a substrate comprising ADP, and the ATP is then readily detected using known methods and reagents. Specific kinases suitable for the invention are set out in SEQ ID NO.s 1-30.

ATP bioluminescent detection is a preferred means of detecting kinase activity. A standard luciferin-luciferase assay method can detect as little as 10^{-15} moles of ATP. By coupling an enzymatic amplification to the bioluminescent detection methods it is possible to detect as few as 10^{-20} moles of kinase. This

type of format therefore offers remarkable sensitivity for the detection of molecules using binding species linked to adenylate kinase (AK) as described in WO 02/053723.

- 5 Use of a kinase, e.g. AK, coupled to bioluminescent detection has a number of other significant advantages. The assay gives a direct relationship between enzymatic activity and light production over a much larger range than other comparable assay formats. Thus whilst an assay using a traditional reporter enzyme such as horseradish peroxidase or alkaline phosphatase will give a
- 10 proportional response over 5-6 log dilutions, the AK-luciferase assay can provide a dynamic range of at least 8 logs. As direct indicators this makes them especially useful for processes which require a level of inactivation greater than the standard 6-log range as the signal can be made to be meaningful across the whole range of the assay, something that would not be
- 15 possible using other assay formats. This is particularly relevant for TSE inactivation where, in a worse case scenario, as many as 8-logs of infectivity may be present on the surface of a surgical instrument, assuming the presence of 1mg brain tissue at a level of up to 10^8 TSE infectious units per mg. Under these circumstances an indicator of the invention, providing an 8-
- 20 log range of signal is particularly valuable.

Given the type of processes for which a TSE indicator is required a high level of both thermal and physical stability is preferred. In an example below, the properties of a range of AK enzymes from thermophilic organisms were

25 compared. Even AKs from thermophilic organisms such as the indicator strain *B.stearothermophilus* lose the majority of their activity at relatively low temperatures. For a kinase-based indicator to be included in e.g an autoclaving cycle, a significantly greater degree of thermostability, such as that demonstrated by the enzymes from the *Sulfolobus* species or *Pyrococcus*

30 *furiosus*, is used.

A number of additives and changes to formulation that increase the stability of an enzyme, e.g. a kinase, to heat inactivation will be known to those familiar with the art. The thermostable kinases used in embodiments of this invention

will require significantly less stabilisation given that they are already significantly more stable than other enzymes used for this type of process. AK enzymes described herein, in particular the AK enzymes from *Sulfolobus acidocaldarius*, *S.solfataricus*, *S.shibatae*, *Pyrococcus furiosus*,
5 *Rhodothermus marinus* and *Thermococcus litoralis*, are significantly more stable at both 80°C and 90°C than even the enzyme from an organism normally used as an indicator of process sterilisation such as *Bacillus stearothermophilus*. In many cases these AK enzymes immobilised on a solid support may require no further stabilisation to provide the necessary range of
10 activity to be measured following e.g. autoclaving, pasteurisation or equivalent.

The addition of stabilising agents such as sorbitol up to a concentration of 4M, or other polyols such as ethylene glycol, glycerol, or mannitol at a
15 concentration of up to 2M may improve the thermostability of the enzyme. Other additives such as xylan, trehalose, gelatin may also provide additional stabilisation effects either individually or in combination. Addition of a range of divalent metal ions, most notably Ca^{2+} , Mg^{2+} or Mn^{2+} may also improve stability of the enzyme.

20 Chemical modification of the enzymes can also be used to improve their thermal stability. Reductive alkylation of surface exposed amino groups by glyoxylic acid (e.g Melik-Nubarov (1987) Biotech letts 9:725-730), addition of carbohydrates to the protein surface (e.g. Klibanov (1979) Anal. Biochem.
25 93:1-25) and amidation (e.g. Klibanov (1983) Adv. Appl. Microbiol. 29:1-28) may all increase the stability of the enzyme. Further methods including the use of chemical cross-linking agents and the use of various polymeric supports for enzyme immobilisation are also relevant methods for increasing the thermostability of enzymes (reviewed in Gupta (1991) Biotech. Appl.
30 Biochem. 14:1-11).

Similar modifications are also relevant to the stabilisation of the indicator against other sterilisation processes such as hydrogen peroxide or ozone. In particular, processes where the access of the gaseous phase sterilant to the

enzyme is restricted, for example by encapsulation in a suitable polymer or formulation with an additive to reduce penetration of the gas, will provide useful methods for increasing the stability of the enzyme if required.

- 5 Many of the treatments that are effective at increasing the thermal stability of enzymes are also relevant to the stabilisation for protease treatments, e.g. for the development of an indicator for the effective inactivation of TSE agents by protease treatment. In general a protein that shows high levels of thermostability is likely to also show a high degree of stability for degradative
- 10 processes such as denaturation or protease treatment (See for example: Daniel RM, Cowan DA, Morgan HW, Curran MP, "A correlation between protein thermostability and resistance to proteolysis", *Biochem J.* 1982 207:641-4; Rees DC, Robertson AD, "Some thermodynamic implications for the thermostability of proteins", *Protein Sci.* 2001 10:1187-94; Burdette DS,
- 15 Tchernajenko V V, Zeikus JG."Effect of thermal and chemical denaturants on *Thermoanaerobacter ethanolicus* secondary-alcohol dehydrogenase stability and activity", *Enzyme Microb Technol.* 2000 27:11-18; Scandurra R, Consalvi V, Chiaraluce R, Politi L, Engel PC., "Protein thermostability in extremophiles", *Biochimie.* 1998 Nov;80(11):933-41; and Liao HH., "Thermostable mutants of
- 20 kanamycin nucleotidyltransferase are also more stable to proteinase K, urea, detergents, and water-miscible organic solvents", *Enzyme Microb Technol.* 1993 Apr;15(4):286-92). The thermostable kinases therefore generally show a higher degree of stability to the actions of the protease treatments designed to inactivate TSE agents than might an equivalent mesophilic kinases.
- 25 Depending on the type of process used, a kinase can also be selected to favour other characteristics of the process. Thus for a protease treatment at alkaline pH the protocol tends towards the use of a thermostable kinase from a moderately alkalophilic organism such as *P.furiosus*, whereas a protease treatment at acidic pH might use a kinase from an acidophile such as
- 30 *S.acidocaldarius* or *S.solfataricus*.

If required to improve the stability of the kinase indicator to protease treatment a number of other options exist. A number of these are the same as those described above for the stabilisation of the enzyme against heat treatment.

For example formulations containing sorbitol, mannitol or other complex polymers reduce the levels of inactivation of the enzyme on the indicator surface. In addition treatments that specifically reduce the rate at which a protease substrate is degraded are particularly relevant to this application. For
5 example the formulation of the kinase in a solution containing up to around 10mg/ml (a 10-fold excess compared to the preferred concentration of the indicator) of a suitable carrier protein such as casein or albumin, that acts as alternative substrate for the protease, will specifically reduce the rate of digestion of the kinase indicator. Similarly the addition of free amino acids
10 such as glycine, tyrosine, tryptophan or dipeptides to the formulation would provide a means of substrate level inhibition of the enzyme and reduce local inactivation of the kinase indicator.

The invention additionally provides methods for the production of various
15 thermostable kinases by recombinant expression in bacteria for use as biological indicators.

The genetic modification of enzymes has been shown to provide significant increases in thermal stability and by analogy such mutations are also likely to
20 significantly enhance the stability of the indicator enzymes in other processes such as protease treatment or gaseous phase "sterilisation". The comparison of the thermostability of the kinase enzymes shown in Figure 1 taken with the defined 3-D structure of the trimeric (archaeal) AKs (Vonnrhein et al (1998) J. Mol. Biol. 282:167-179 and Criswell et al (2003) J. Mol. Biol. 330:1087-1099)
25 has identified amino acids that influence the stability of the enzyme.

Genetically engineered variants of kinases showing improved thermostability are also provided by and used in the invention, and can be generated in a number of ways. Essentially these involve the specific site-directed
30 mutagenesis of amino acids believed to form part of the central core packing region of the trimeric molecule and random "directed evolution" methods where the whole molecule is subjected to subsequent rounds of mutagenesis and selection/screening of molecules with improved properties. The modifications outlined herein, e.g. in Examples 8-10, are based on a hybrid

approach using a consensus based approach to define regions likely to influence the thermostability of the enzymes based on observed differences between structurally related molecules. This is followed by either defined changes to incorporate the amino acids that correlate with the best
5 thermostability or a random replacement to incorporate every available amino acid at the positions defined as being essential for thermostability.

Specific modified enzymes of the invention are the various variants set out in Examples 8-10 and referred to collectively as SEQ ID NOs: 17-19 (though
10 several variants are embraced by each reference).

The biological indicator can be adapted for use in a vessel in which an sample is being processed. For example, the indicator includes a solid support which is a matrix and the kinase is dispersed within the matrix. The matrix should be
15 resistant to the process conditions and can help to provide protection to the kinase from the process – hence providing some stabilization to the indicator. The kinase can be located within a polymer matrix. The support can be designed to project into or be added to a processing vessel, and can be an indicator strip, a dip stick or a bead.

20 A variety of solid supports, with or without chemical modifications and with one or more kinase indicators in a variety of formulations, are embraced by the invention, depending e.g. on the requirements of the process to be validated. In one form the support is a plastic, ceramic, steel or other metallic
25 or polymer surface onto which the kinase is dried as a means of immobilisation. The support can be a polycarbonate, polystyrene or polypropylene strip or dipstick, possibly with a flattened surface, onto which the kinase is applied. An additional type of support with a porous surface for attachment of kinase is also particularly useful as an indicator for gaseous
30 processes. Kinase coated plastic, metallic or ceramic beads may also provide a valuable format for the indicator, again with specific relevance to monitoring gaseous processes. Such supports have advantages over solid supports for certain applications, as they provide a significantly increased surface area for the attachment of the indicator.

For the specific example of a device formulated for the validation of TSE inactivation processes, a steel surface such as a rod, disk or coupon is an effective indicator for surgical instrument decontamination. The binding of a

5 thermostable adenylate kinase, for example, is a very good model of the aggregated form of the disease-related prion isoform (PrP^{Sc}) and as such provides a remarkably good indicator of PrP^{Sc}'s interaction with the surface of surgical steel.

10 Binding of kinase on or to the support of the indicator may be achieved by methods routinely used to link protein to surfaces, e.g. incubation of protein in 0.1M sodium bicarbonate buffer at about pH9.6 at room temperature for about 1 hour. Alternatively the protein is covalently coupled to the surface using any of a wide range of coupling chemistries known to those familiar with the art.

15 For example an adenylate kinase derivitised with SPDP (Pierce chemicals; using manufacturer's instructions), reduced with DTT to provide free sulfhydryl groups for cross-linking, is covalently attached to a polystyrene support with a maleimide surface. Plastic surfaces with such sulfhydryl-binding surfaces are well described in the literature. An added benefit of this method of coupling is

20 that, if required, the enzyme can be cleaved from the support eg. by reduction with DTT or MESNA, to allow the assay to be carried out separately to any indicator support. The adenylate kinase enzymes and other indicator kinases described by this invention have the property that their activity is retained upon derivitisation and cross-linking to such supports.

25 Alternatively an amine reactive surface on a polystyrene or polycarbonate support is used, with a bifunctional cross-linking agent such as monomeric glutaraldehyde, to provide direct non-cleavable cross-linking of the kinase via free amine groups on the protein. UV treatment can also be used to directly

30 link the indicator to a suitable support. Steel surfaces can be treated in a similar way to plastic surfaces to mediate covalent attachment of the indicator kinase.

A wide variety of protein cross-linking reagents are available from companies such as Pierce chemical company (Perbio). Reagents reactive to sulfhydryl, amino, hydroxyl and carboxyl groups are designed for coupling proteins but they can equally be used for cross-linking proteins to either naturally reactive or coated solid supports such as plastics, other polymers, glass and metals. Reactive chemistries are also available for cross-linking the enzymes to carbohydrates. For example, the reagents BMPH ((N-[β -Maleimidopropionic acid]hydrazide•TFA), KMUH ((N-[γ -Maleimidoundecanoic acid]hydrazide), and MPBH (4-(4-N-Maleimidophenyl)butyric acid hydrazide hydrochloride) can be used to cross link the kinase containing either a free sulfhydryl in the form of a cysteine residue or a chemically derivitised protein reduced to generate a sulfhydryl reactive group, to carbohydrates. This may be particularly important for a solid support which is either a complex carbohydrate (e.g. paper, cellulose-based membranes, gels or resins) or can be coated or treated with a carbohydrate solution to generate a suitably reactive surface.

For each type of support the kinase is preferably formulated in a solution that enhances binding and/or stabilises the bound protein. Such formulations include solutions containing up to 10% (w/v) sucrose, sorbitol, mannitol, cellulose, or polyethylene glycol (PEG). In addition the kinase can be formulated as part of a gel that is applied to the surface or lumen of a suitable support. Examples include alginate, agar or polyacrylamide matrices.

The indicator may also comprise an agent to stabilise the kinase, and suitable stabilising agents are selected from metal ions, sugars, sugar alcohols and gel-forming agents.

To facilitate use of the indicator, it may further comprise means to attach the indicator to a surface, such as a projection, recess or aperture for attachment of the support to a surface by means of a screw, nut and bolt or clamp.

In specific embodiments of the invention, purified kinase, e.g. adenylyate kinase (AK), is formulated at a concentration of up to 1mg/ml and coated onto solid supports. Preferably, between 1-2mg, or 0.5-1mg, or 0.1-0.5mg, or

0.1mg, of kinase is coated onto the solid support. For protease treatment, the kinase may be dried onto a polypropylene, polycarbonate or polystyrene surface similar to microtitre plates. For either standard autoclaving at 121°C for 15-20 minutes or "prion-cycle" autoclaving at 134°C for 18 minutes, a heat-stable support such as stainless steel may be used. For gas phase inactivation procedures such as hydrogen peroxide or ozone, polycarbonate solid support may be used, and can also be manufactured as a porous matrix to provide a greater degree of resistance to the inactivant if required.

- 10 A convenient solid support takes the form of a dipstick which is transferred directly from the inactivation procedure to a tube containing all the required assay components. This can be a tube luminometer attached to one of a range of "rapid read-out" hygiene monitors already on the market for the food and pharmaceutical industry. Alternatively it can take the form of a specialised instrument designed for the indicator in question, with a particular emphasis on maintaining the optimal temperatures required by the thermostable enzymes (see Example 24).

The present invention also provides a biological indicator comprising a plurality of enzymes detectable after differing levels of inactivation of the biological agent. A biological indicator of one embodiment comprises a support, a first enzyme located at a first position and a second enzyme located at a second position, separate from the first position. Both the first and second enzymes have activity in converting product to substrate and after exposure of the biological indicator to an inactivation process for an initial period of time activity of both enzymes can be detected. After exposure of the biological indicator to the inactivation process for a subsequent period of time, activity of the first enzyme cannot be detected but activity of the second enzyme can be detected. After exposure of the biological indicator to the inactivation process for a second subsequent period of time, activity of the second enzyme cannot be detected.

An advantage of this embodiment is that the indicator can be used to show an approximate level of inactivation of the biological agent achieved by the

process without the need for a precise measurement to be taken. Thus, for example, when both enzymes are detectable this can indicate that the inactivation has not reached a certain threshold. When only the second enzyme can be detected this indicates that the first threshold of inactivation has been reached but a second threshold has not. Lastly, when neither enzyme can any longer be detected this indicates that the inactivation has passed the second threshold. If the first enzyme is detectable at up to 6 logs reduction in activity and the second enzyme is detectable at up to 8 logs reduction in activity, then being able to detect both enzymes indicates that inactivation has not reached 6 logs, being able to detect only the second enzyme indicates activity has been reduced by between 6 and 8 logs and being able to detect neither indicates that at least 8 logs reduction in activity has been achieved.

The first enzyme is suitably detectable at up to between 5 and 8 logs reduction in activity and the second enzyme is suitably detectable at 6 logs or greater reduction in activity. The first enzyme is preferably detectable at between 6 and 7 logs reduction in activity and the second enzyme is preferably detectable at between 7 and 8 logs reduction in activity. The biological indicator may further comprise a third enzyme located at a third position (separate from the first and second positions), wherein after exposure of the biological indicator to the inactivation process for the second subsequent period of time, the third enzyme can be detected, and after exposure of the biological indicator to the inactivation process for a third subsequent period of time, the third enzyme cannot be detected. The third enzyme is suitably detectable at 8 logs reduction in activity or greater.

Using a biological indicator of this type, having multiple enzymes that are detectable at differing levels treatment (e.g. a different exposure time), the progression of the inactivation progress can be watched and its end point anticipated readily.

The preferred enzymes of the multi-enzyme indicator are kinases, more preferably thermostable and more preferably as disclosed and described herein in relation to other embodiments of the invention.

- 5 In a second aspect of the invention, there is provided a kit (optionally a portable kit) for use in validating a treatment process for reducing the amount or activity of a biological agent in a sample, comprising:

- 10 (i) a biological process indicator according to the first aspect of the invention, and
(ii) substrate for the kinase.

To carry out measurement of the kinase amount / activity, the kit can include means for detecting ATP, e.g. luciferin/luciferase and optionally a
15 luminometer. The substrate is preferably ADP.

From previous testing with known biological agents, data correlating reduction in the amount or activity of the biological agent with kinase activity can be prepared, and the kit therefore can also include one or more look-up tables
20 correlating kinase activity with the reduction in amount or activity of a list of specified biological agents. In preferred embodiments, the kit is for monitoring TSE inactivation.

In a third aspect of the invention, there is provided a method of validating a
25 treatment process, comprising:

- (i) obtaining a sample that contains, or is suspected to contain, a biological agent;
(ii) subjecting the sample to a treatment in the presence of a
30 defined amount of a kinase, wherein the treatment reduces the amount or activity of the biological agent;
(iii) measuring residual kinase activity and optionally calculating the reduction in kinase activity; and

- (iv) comparing said residual activity to a predetermined kinase activity, or comparing said reduction in kinase activity to a predetermined reduction in kinase activity, wherein the predetermined kinase activity or predetermined reduction in kinase activity corresponds to a confirmed reduction in the amount or activity of the biological agent under the same treatment conditions.

In this context, the kinase may be any one of, and/or have any of the properties of, the kinases described in this specification. Preferably, the kinase is formulated as an indicator according to the first aspect of the invention.

The sample generally is provided without any kinase present, so the method may comprise obtaining a sample believed to contain the biological agent and adding a defined amount of kinase. The agent may not be present at all (although preferably the sample is known to contain the biological agent). The point of the validation is that, after carrying out the treatment, it is confirmed that any agent that might have been present has been removed/inactivated to an acceptable degree.

Typically, an operator measures kinase activity prior to treating the sample and after treating the sample. It is also possible that contaminating, usually mesophilic, kinase can get into the sample prior to assaying for kinase activity. It is thus preferred that the kinase that is added to the sample is thermostable and that the assay step includes inactivating mesophilic kinase, such as by treating the sample at 70 degrees C for at least 30 minutes, preferably 80 degrees C for at least 10 minutes, prior to measuring residual kinase activity.

In preferred embodiments, the kinase, prior to the treatment, has an activity of at least 10,000,000 Relative Light Units (RLU) per mg kinase, or at least 8,000,000 RLU per mg kinase, or at least 5,000,000 RLU per mg kinase, or at least 3,000,000 per mg kinase, or at least 1,000,000 RLU per mg kinase, or at

least 500,000 RLU per mg kinase, when measured in the presence of luciferin/luciferase by a luminometer.

5 In preferred embodiments of the invention, the predetermined kinase activity is less than 10,000 RLU per mg kinase, or less than 1000 RLU per mg kinase, or less than 500 RLU per mg kinase, or less than 250 RLU per mg kinase, or less than 100 RLU per mg kinase, or less than 10 RLU per mg kinase, or less than 1 RLU per mg kinase, or is 0 RLU per mg kinase.

10 In preferred embodiments of the invention, the predetermined reduction in kinase activity is equal to or greater than a 1 log reduction, or a 2 log reduction, or a 3 log reduction, or a 4 log reduction, or a 5 log reduction, or a 6-log reduction, or a 7 log reduction, or an 8 log reduction or a 9 log reduction in kinase activity.

15

In other embodiments, the predetermined reduction in kinase activity corresponds to at least a 6 log reduction, or a 7 log reduction, or an 8 log reduction, or a 9 log reduction, in the amount or concentration of the kinase. In further embodiments, the predetermined reduction in kinase activity
20 corresponds to a reduction in RLU of at least 800,000, or at least 900,000, or at least 950,000, or at least 990,000, or at least 999,000, or at least 999,900, or at least 999,990, or at least 999,999 RLU.

25 In preferred embodiments of the invention, the confirmed reduction in the amount or activity of the biological agent within the sample is at least 6 logs, preferably at least 7 logs, more preferably at least 8 logs, most preferably at least 9 logs.

30 In particularly preferred embodiments, the treatment is continued until the residual kinase activity or the reduction in the kinase activity corresponds to a confirmed reduction in the amount or activity of the biological agent of at least 6 logs, or at least 7 logs, or at least 8 logs, or at least 9 logs.

In a fourth aspect of the invention, there is provided a method of correlating the reduction in the amount or activity of a biological agent in a sample with the kinase activity of an indicator according to the first aspect of the invention, comprising:

5

- (i) preparing a sample containing a defined amount of the biological agent and a sample containing a defined amount of the kinase;
- (ii) subjecting the sample(s) to a treatment;
- (iii) measuring the residual activity of the kinase and optionally calculating the reduction in kinase activity;
- (iv) measuring residual amount or activity of the biological agent and optionally calculating the reduction in the amount or activity of the biological agent;
- (v) repeating steps (i) to (v), wherein at least one of the treatment parameters is changed.

10

15

In one embodiment, the biological agent and the kinase may be present in the same sample.

20 In a preferred embodiment, the treatment parameter comprises one or more of time, temperature, pH, pressure, protease concentration, and concentration of sterilant or detergent.

In a particular embodiment, the treatment comprises heating the sample(s) at 25 50-140°C, preferably 80-100°C, more preferably 134-138°C; the treatment parameter is time; and steps (i) to (iv) are repeated by subjecting the sample(s) to said treatment for periods of 1, 5, 10, 20, 40 and 60 minutes.

In a further embodiment, the treatment comprises exposing the sample(s) to a 30 pH of 9-14, preferably pH 12 or above, more preferably about pH12; the treatment parameter is time; and steps (i) to (iv) are repeated by subjecting the sample(s) to said treatment for periods of 1, 5, 10, 20, 40 and 60 minutes.

In another embodiment, the treatment comprises exposing the sample(s) to a protease at a concentration of 0.5-2 mg/ml, preferably about 1mg/ml, more preferably about 2mg/ml; the treatment parameter is time; and steps (i) to (iv) are repeated by subjecting the sample(s) to said treatment for periods of 1, 5,
5 10, 20, 40 and 60 minutes.

The above method enables preparation of calibration data for future use of the indicator for validation of a treatment on samples containing, or suspected of containing a biological agent. The calibration of a number of treatment
10 processes is described in Examples 21-23.

In a fifth aspect of the invention, there is provided use of a kinase, as an indicator for validating a treatment process for reducing the amount or activity of a biological agent in a sample. In this context, the kinase may be any one
15 of, and/or have any of the properties of, the kinases described in this specification. Preferably, the kinase is formulated as an indicator according to the first aspect of the invention.

The use of the indicator of the invention to monitor/validate a variety of
20 processes is now described.

In one embodiment, the indicator is used to validate the performance of a biological washing preparation in a wash cycle (see Example 14). Whilst validation of a wash cycle would potentially be of use in a domestic setting, its
25 most advantageous use would be within a healthcare, pharmaceutical or food preparation setting, e.g. for validating decontamination of bedclothes, gowns or other items associated with patients suffering or exposed to infectious agents (e.g. an outbreak of methicillin resistant *Staphylococcus aureus* (MRSA) or Norwalk/Norwalk-like virus). In this context, the indicator of the invention
30 has the advantage that it is relevant to biological material such as blood or other bodily fluids.

For the validation of a wash cycle, the indicator is prepared by cross-linking a suitable kinase onto a flexible wand, strip of cloth or other material suitable for

inclusion within the cycle. The indicator is put into the washer with the remainder of the load. Preferably, the indicator may be fixed within a suitable holder on the inside of the washer to facilitate its recovery.

- 5 The wash cycle is then performed and the indicator removed and assessed prior to any further handling or processing of the load, using a “reader” which has been calibrated to indicate an acceptable level of residual kinase activity within the indicator – the acceptable level having been derived from previous calibration and assessment of suitable wash performance within the process.
- 10 Such assessment might include the overall levels of soiling and the viable count of micro-organisms as assessed using suitable model organisms known to those familiar with the art. Based on the calibrated read-out, the load is passed for further processing or the wash cycle is repeated.
- 15 In a second embodiment, the indicator is used to validate processes for the inactivation of viruses (see Example 15). The detection of live viral isolates in the environment is problematic, particularly when associated with an emergency situation where speed and accuracy may be critical. The present invention provides the possibility of developing indicator systems that allow
- 20 the monitoring of decontamination procedures essentially in real time. This would be particularly valuable for surface decontamination in healthcare and related facilities following either an outbreak (e.g. of Norwalk-like viruses) or a deliberate release of a viral agent (such as small pox).
- 25 An indicator for validating a viral inactivation process can take a variety of different forms, e.g. a wand or dipstick for monitoring an area sprayed or immersed with virucide, or a suspended indicator for monitoring a gaseous phase decontamination process. Alternatively, the indicator kinase can be sprayed onto a surface prior to decontamination and the levels of residual
- 30 kinase activity subsequently assessed by swabbing of the surface.

In a further embodiment of the invention, the indicator is used for validating protease degradation of bacterial protein toxins, plant toxins such as ricin, and other toxic proteins, peptides, or peptide analogues (see Example 16).

Proteases show significant potential for the degradation of a wide range of protein toxins that are potential biowarfare/bioterror threat agents including botulinum toxin, anthrax toxins and ricin. They also have the potential to inactivate a wide range of other potentially toxic or harmful protein or peptide agents to enable decontamination of surfaces/facilities or the safe disposal of materials. In this context, the indicator of the invention, together with the surface/material to be decontaminated, are subjected to the protease decontamination procedure. At the end of the procedure, the indicator is removed and the level of residual kinase activity assessed according to the method of the invention. The level of residual kinase activity is then correlated with inactivation indices for the particular protein toxin, or group of toxins. Assuming the level activity is equal to or below the defined index value then the material can be safely disposed of or the surface/facility returned to use.

Preferably a suitable safety margin is built into the calibration of the inactivation indices to allow for any variability of the process performance. The additional stability of the enzymes used in this invention allow for this to be done with more certainty and greater dynamic range than a wide range of other enzymatic indicators, including those from "thermostable" organisms such as *Bacillus stearothermophilus*, as shown by the data showing the relative thermal stability of AKs from thermophilic organisms (Figure 1, Example 2).

The indicator may also be used to validate protease decontamination procedures for cleaning down pharmaceutical production apparatus. A wide variety of pharmaceutical products use materials from either humans, or animals that might be contaminated with a wide variety of agents including prion (TSE) agents and viruses (e.g. West Nile virus, hepatitis, HIV). The risks may be exacerbated when the source of the material is of animal origin (e.g. foetal calf serum, horse immunoglobulins) and where an intermediate processing stage may carry the risk of increasing the concentration of unidentified pathogens in a particular sample. The possibility of using a protease to clean down manufacturing facilities and apparatus (e.g.

chromatography columns, vessels, pipework) between manufacturing batches has the potential to reduce or eliminate such risks, even when the contaminant has not been formally identified. This is particularly true for prion agents in, for example, blood fractionation apparatus where there is a significant risk of accumulation and of carrying an infection risk into the final product.

For validating this type of procedure, the indicator of the invention is ideally designed as a dipstick to be immersed in the protease treatment solution, or as a cartridge to be attached in line with the apparatus to be cleaned. By assessing the levels of residual kinase activity in the indicator device following the treatment, and correlating this with the acceptable levels of cleaning, a rapid and reliable monitor of performance can be developed.

In another embodiment of the invention, the indicator is used for validating gas phase inactivation of biological agents, such as TSE (see Example 17).

The potential of ozone or other gas phase sterilants to inactivate such agents is suggested by a wide range of publications and articles, however, as yet, no method has explicitly been shown to be effective. To support the development and introduction of this gas phase technology into healthcare, a means of validating the performance of the technology will be required. As agents such as TSE have already been shown to be far more resistant to this form of inactivation than conventional viral or bacterial agents, the methods currently available for validating gas phase inactivation are unlikely to be suitable. The present invention addresses this problem.

For this type of validation, the indicator kinase is attached onto a solid support by any suitable method, e.g. general adsorption and chemical cross-linking via amide, peptide, carbonyl, or cysteine bonds. For example, for ozone sterilisation, a rigid polyvinyl chloride (PVC), glass, steel, polyamide or polypropylene support may be used, with the kinase coupled to the support by any one of the chemical methods previously described. The indicator is then included in the batch of material/instruments to be sterilised, exposed to the

ozone, and assessed against a suitably calibrated inactivation index designed for assessing corresponding inactivation of the agent in question. Successful inactivation allows onward processing or use of the material/instruments.

- 5 The indicator may optionally be attached to the internal face of a tube or equivalent internal space, such that the penetration of the gas is restricted. This provides for a monitor that is suitable for assessing the penetration of the gas into equivalent spaces in instruments with lumens, or through packed loads of material. Alternatively, the kinase may be attached to porous
10 materials such as polystyrene beads, or may be immobilised within a gel or resin.

In a further embodiment of the invention, the indicator is used for validating liquid chemical sterilisation systems (e.g. Endoclens) as used for processing
15 of endoscopes and related equipment (see Example 18).

A wide range of endoscopes are routinely used in medicine and are an important part of medical diagnosis and treatment. These instruments are extremely sensitive and have posed a very significant problem for routine
20 cleaning and disinfection. Traditionally, and remaining in current practice, endoscopes are cleaned by hand before being decontaminated using a low temperature method. A range of chemical disinfectants and automated re-processing apparatus has been developed to address the specific issues of decontaminating sensitive pieces of equipment such as endoscopes, where
25 traditional autoclaving is not possible. These methods have helped to reduce the levels of contamination on difficult to clean instruments, which have been associated with the iatrogenic transmission of a wide range of viral and bacterial pathogens. The current method of validating such processes is to monitor the flow rate and temperature of the washing solution. The indicator of
30 the invention provides for a further means of validation that provides a read-out of actual cleaning effectiveness within the endoscope lumen.

For this type of validation, the indicator is attached to the internal surface of a tube designed to be of a similar overall internal diameter to the endoscope

tube. This indicator apparatus is connected in series to the endoscope on the automatic reprocessing apparatus. The endoscope is then processed in the normal way. At the end of the process, preferably before the endoscope is removed from the apparatus, the indicator is detached and assessed for the level of kinase activity remaining. The level of activity may be correlated with previously defined thresholds for the acceptable performance of the process and, based on this assessment, the endoscope may be transferred for additional cleaning or decontamination or prepared for use. If the level of performance is not adequate then the instrument may be re-processed (using the same or more stringent conditions) with a new indicator attached as previously. The indicator apparatus is also suitable for validating the manual cleaning of endoscope and/or any other instrument with a lumen.

In preparing indicators for the above validation, it is preferred that the kinase adheres to its support via non-specific protein adsorption methods based on the hydrophobicity of the enzyme. Figure 5 demonstrates the ability of different recombinant kinases to adhere to polystyrene in this way. Figure 5 also demonstrates that certain kinases have more desirable properties for this type of application, e.g. the *S.acidocaldarius* kinase is significantly more adherent to this type of surface when compared with kinases from *T.maritima* and *A.fulgidus*. This may be different for other types of surface, especially where the attachment to the surface may be via charged amino acid residues. In this type of indicator, the straightforward removal of the kinase from the surface is the determining feature in assessing cleaning performance, rather than any change or modification to the kinase. As such, this type of "adsorbant" indicator has a very wide range of uses in assessing washing performance. This is particularly important for validating the ability of the process to effectively remove bio-films from the lumen of devices such as endoscopes as this is thought to be one of the key factors affecting performance. As such, a suitable non-infectious biofilm may be used as a matrix for the kinase, preferably using serum, sucrose solution, aqueous gel or other means of simulating a biofilm to ensure that the process is effective against this form of contamination. Other types of indicator suitable for validating such processes are similar to those previously described, where the

kinase is covalently attached to the support surface to generate chemical linkages such as disulfide, amide or carbonyl bonds. In these cases, the effects of the wash and/or sterilant are exerted by modifying the kinase so that it is no longer active. This would be particularly relevant for chemical sterilants
5 that work by breaking peptide bonds, cross-linking proteins or related methods that perturb the structure of proteins.

In a further embodiment of the invention, the indicator is used to monitor routine cleaning performance in washer-disinfectors, such as those used in
10 hospitals (see Example 19).

In another embodiment of the invention, the indicator is used for monitoring glutaraldehyde or ortho-phthalaldehyde (OPA) treatments. Glutaraldehyde and formaldehyde have been widely used as sterilants over many years. The
15 chemical disinfectants work by multiply crosslinking proteins in a non-specific fashion to destroy their function. Ortho-phthalaldehyde (OPA) has emerged recently as a new disinfectant in this family and is being widely used as it avoids some of the toxicity problems associated with glutaraldehyde. The indicator of the invention is suitable for the monitoring of all of this class of
20 chemical disinfectants as the kinases are sensitive to non-specific crosslinking of this kind. In this type of indicator, the kinase is covalently attached to a suitable surface and exposed to the chemical sterilant along with the other items to be sterilised. The effectiveness of the process is assessed by removing the indicator and measuring residual enzyme activity. This activity is
25 compared to defined threshold values that indicate the correct performance of the process.

The use of different types of kinase may provide additional sensitivity or susceptibility to the process as may be required for different applications. The
30 thermostable adenylate kinases described in this specification can be broadly classified into two groups based on their molecular architecture. Thus the enzymes from *Sulfolobus* species are examples of enzymes that have a trimeric structure with a central hydrophobic core that is the principle determinant in maintaining their activity at high temperatures. The second

group of enzymes are monomeric, exemplified by the adenylate kinases from *Thermotoga* species, but have a slightly longer polypeptide chain with an additional "lid" domain that affects the active site. These different types of thermostable enzymes will show differential sensitivity to this type of chemical
5 sterilant due to the variable flexibility of their peptide chains during enzyme action. For any particular sterilant and/or concentration an empirical screen will identify enzymes with suitable susceptibilities for monitoring and validating these types of chemicals.

- 10 In a further embodiment of the invention, the indicator is used as an ultra-rapid read-out monitor for ethylene oxide, hydrogen peroxide or other gas phase processes.

A wide range of gas phase sterilants are currently being used by a variety of
15 manufacturers for routine disinfection of bacterial and viral agents. The current methods exploit the oxidative properties of the gases to destroy peptide linkages. As such, the kinases of the present invention, with their robust physicochemical properties, are ideal for providing a very rapid read-out of inactivation. The indicator in this example is similar to those described
20 previously, e.g. in relation to the ozone inactivation of agents such as TSE.

A particularly challenging issue for sterilisation and decontamination processes is the ability to validate sterility of large bulk liquids, as might be required in the manufacture of various medicines or other pharmaceutical
25 products. Whilst current methods monitor the temperature, time, and/or pressure parameters of a particular process (depending on its precise nature), there are few, if any, available methods for validating actual sterilisation within the bulk liquid. This is difficult even within volumes of around 1 litre, but is almost impossible at larger volumes.

30

The present invention provides a number of possible solutions to address this problem (see Example 20). In its simplest form, the kinase may be added to the liquid to be sterilised at a concentration suitable for measuring defined levels of kinase inactivation at the end of the process and equating this to

levels of sterilisation. Whilst this might not be desirable in certain types of processes, the inert nature of the kinase and the ubiquitous presence of equivalent enzyme activities in all organisms, may make it acceptable. The acceptability may be improved by the fact that many thermostable enzymes
5 are highly condensed and thus have very low immunogenicity following inoculation into animals.

Where such direct additions are not acceptable, the kinase may be added to the bulk liquid in a dialysis sack, porous container or immobilised to a suitable
10 support such that no part of the enzyme is released into the bulk liquid, but the sterilising conditions work on the indicator in the same way as for the whole sample. A wide variety of possible ways of containing or immobilising proteins, to allow general diffusion of the liquid sample but to restrict the movement of the indicator sample, will be known to those familiar with the art. Possible
15 examples include, but are not limited to dialysis membranes, Visking tubing, porous membranes, protein-binding resins, rigid gels or solid supports as described for the other indicators discussed. The indicator may be attached to the surface by any one of the methods discussed previously, or simply encased within a suitable membrane without attachment, such that the
20 indicator may be simply removed from the bulk liquid at completion of the process.

Definitions Section

25 The term "biological agent" encompasses both infectious and non-infectious agents. Specific examples of biological agents relevant to the present invention include bacteria, viruses, spores, proteins, peptides and prions (both the infectious PrP^{Sc} form and the non-infectious PrP^C form) and also the specific agents mentioned in Examples 14-20. In preferred embodiments of
30 the invention, the biological agent is a transmissible spongiform encephalopathy.

The term "treatment" or "treatment process" encompasses any process that is designed to reduce the amount or activity of a biological agent in a sample.

- Suitable treatments include one or more of: a selected pH, temperature or pressure, exposing the sample to a protease or other enzyme, exposing the sample to a detergent, a chemical sterilant or a gas-phase sterilant. In a preferred embodiment, the treatment is designed to reduce the infectious activity (also known as the infectivity) of an infectious biological agent, such as TSE. In particularly preferred embodiments, the treatment comprises exposing the sample to a protease at a temperature of between 50-120°C, preferably 60°C or above, more preferably 100°C or above, and/or exposing the sample to a pH of at least 9, preferably at a pH of at least 12. The term "treatment" also encompasses cleaning and inactivation processes such as high temperature autoclaving with wet or dry steam, ozone sterilisation, H₂O₂ sterilisation, rendering or other method designed to eliminate or inactivate the biological agent.
- The term "sample" encompasses any item, instrument, surface, fluid or material. Examples include, but are not limited to, surgical and medical instruments, hospital gowns, bedclothes, bulk liquids, culled animal material, pharmaceuticals, workbenches, walls and floors.
- The term "RLU" means Relative Light Unit. Those familiar with the art will recognise that Relative Light Units are a relative, not absolute, measurement. The figures given in the specification relate to measurements taken using a Berthold Orion 96-well microplate luminometer with injector system using a "flash" method of light measurement for 2 seconds immediately after the addition of the luciferase/luciferin reagents (technical specification photomultiplier measuring light emitted at a wavelength of 300-650nm). To address this issue, manufacturers have generated data for RLU "factors", which allow the data generated by a given luminometer to be normalised to a calibrated standard. Thus, comparisons can be made between different instruments. The RLU factor for the Berthold Orion 96-well microplate luminometer used in the experiments described in the present specification is 1. Accordingly, the RLU values given in the specification can be regarded as standardised/normalised RLU values.

In terms of absolute values, an RLU value can be related to the concentration of ATP required to give said value with the reagents as described in the method (e.g. of Example 1). As an approximate conversion, and given the linear relationship between RLU values and ATP concentration, the following

5 values can be used:

| RLU | Approximate concentration of ATP / μM |
|------------|--|
| 12,000,000 | 1000 |
| 1,200,000 | 100 |
| 120,000 | 10 |
| 12,000 | 1 |
| 1,200 | 0.1 |
| 120 | 0.01 |

The invention is now described in specific embodiments in the following examples and with reference to the accompanying drawings in which:-

10

Figure 1 shows activity of adenylate kinase (AK) enzymes after treatment at 70°C (A), 80°C (B) and 90°C (C);

Figure 2 shows residual enzyme activity after digestion of adenylate kinase (AK) with differing concentrations of alkaline protease; and

15

Figure 3 shows standard curves correlating enzyme activity with residual concentration of adenylate kinase (AK).

Figure 4 shows the thermal stability of a range of AK enzymes recombinantly expressed in *E.coli*. Genes encoding AK enzymes were cloned and expressed as described in Example 4. All genes were expressed from the vector pET28a except for *S.acidocaldarius* clone I which was expressed from pET3a as described previously. Expression levels were similar for each clone

20
25

but a proportion of the *Pyrococcus furiosus* (P.fu) enzyme was in the insoluble

fraction and this is likely to have reduced the amount of this enzyme being assayed. The thermal stability of the recombinant enzymes was measured following incubation at 80°C for 30 minutes in a crude *E.coli* lysate at 10-fold serial dilutions from 1 mg/ml total cellular protein (such that column 12 is equivalent to 1 fg/ml total protein). Enzymes from *Thermotoga maritima* and *Archaeoglobus fulgidus* showed significantly greater stability than the other enzymes tested, although the remaining enzymes (*Sulfolobus solfataricus* (S.so P2), *Aeropyrum pernix* and P.fu) showed similar activity to the *S.acidocaldarius* enzyme used as the basis of previous assays (data labelled as S.ac I).

Figure 5 shows the differential binding of thermostable adenylate kinases (tAK) to solid supports. To assess the relative binding of purified recombinant thermostable adenylate kinases to solid supports, demonstrating their potential use for direct adsorbance type indicators, the binding of the enzyme to blocked plates was assessed. The surface of a standard polystyrene microtitre plate was blocked by the addition of 5% skimmed milk. The milk was removed and dilutions of tAKs from *Archaeoglobus fulgidus* (Afu), *Thermotoga maritima* (Tma) and *Sulfolobus acidocaldarius* (Sac) were applied to the plate. After washing, the amount of bound tAK was measured as described for the standard assay method (Example 1). The results demonstrate that the Sac tAK showed significantly higher binding to the blocked plate than either of the two alternative tAKs. Whilst the specific activity of the enzymes were not identical, both Afu and Tma tAKs showed higher activity than that from Sac, such that the real difference in binding is even more exaggerated than shown in the figure. As skimmed milk is a commonly used blocking agent for reducing protein binding to plates these results demonstrate that the Sac tAK has an extremely strong tendency for hydrophobic adsorption to the surface used in these experiments (polystyrene). This property of the enzyme, similar to that observed for the prion molecule to steel surfaces means that it is an extremely effective indicator for assessing a wide range of TSE inactivation and/or removal processes.

Figure 6 shows the preparation of a calibration curve for a protease digestion treatment for inactivating prions.

Figure 7 shows the preparation of a calibration curve for a gas-phase
5 ozone treatment for inactivating prions.

Figure 8 shows the preparation of a calibration curve for a hospital washer-disinfector for removing standard soiling.

10 **Figure 9** shows the modification of a hand held hygiene monitor to allow rapid read-out assessment of tAK indicators.

15

20

25

30

SEQ ID Nos

| | | |
|----|------------------|--|
| 5 | SEQ ID 1 | Protein sequence of Adenylate kinase from <i>Sulfolobus solfataricus</i> |
| | SEQ ID 2 | Protein sequence of Adenylate kinase from <i>Sulfolobus acidocaldarius</i> |
| 10 | SEQ ID 3 | Protein sequence of Adenylate kinase from <i>Sulfolobus tokodaii</i> |
| | SEQ ID 4 | Protein sequence of Adenylate kinase from <i>Pyrococcus furiosus</i> |
| 15 | SEQ ID 5 | Protein sequence of Adenylate kinase from <i>Pyrococcus horikoshii</i> |
| | SEQ ID 6 | Protein sequence of Adenylate kinase from <i>Pyrococcus abyssi</i> |
| 20 | SEQ ID 7 | Protein sequence of Adenylate kinase from <i>Methanococcus thermolithotrophicus</i> |
| | SEQ ID 8 | Protein sequence of Adenylate kinase from <i>Methanococcus voltae</i> |
| 25 | SEQ ID 9 | Protein sequence of Adenylate kinase from <i>Methanococcus jannaschii</i> |
| 30 | SEQ ID 10 | Protein sequence of Adenylate kinase from <i>Methanopyrus kandleri</i> |
| | SEQ ID 11 | Protein sequence of Adenylate kinase from <i>Methanotorris igneus</i> |

| | | |
|----|------------------|---|
| | SEQ ID 12 | Protein sequence of Adenylate kinase from <i>Pyrobaculum aerophilum</i> |
| 5 | SEQ ID 13 | Protein sequence of Adenylate kinase from <i>Thermotoga maritima</i> |
| | SEQ ID 14 | Protein sequence of Adenylate kinase from <i>Aeropyrum pernix</i> |
| 10 | SEQ ID 15 | Protein sequence of Adenylate kinase from <i>Archaeoglobus fulgidus</i> |
| | SEQ ID 16 | Protein sequence of Adenylate kinase from <i>Pyrococcus abyssi</i> (monomeric adenylate kinase (AdkE)) |
| 15 | SEQ ID 17 | Protein sequence of Adenylate kinase from <i>Pyrococcus furiosus</i> genetically engineered to provide improved stability |
| 20 | SEQ ID 18 | Protein sequence of Adenylate kinase from <i>Pyrococcus horikoshii</i> genetically engineered to provide improved stability |
| | SEQ ID 19 | Protein sequence of Adenylate kinase from <i>Sulfolobus acidocaldarius</i> genetically engineered to provide improved stability |
| 25 | | |
| | SEQ ID 20 | Protein sequence of Acetate kinase from <i>Thermatoga maritima</i> |
| 30 | | |
| | SEQ ID 21 | Protein sequence of Pyruvate kinase from <i>Pyrococcus horikoshii</i> |

SEQ ID 22 Protein sequence of Pyruvate kinase from *Sulfolobus solfataricus*

SEQ ID 23 Protein sequence of Pyruvate kinase from *Thermotoga maritima*

SEQ ID 24 Protein sequence of Pyruvate kinase from *Pyrococcus furiosus*

SEQ ID 25 Protein sequence of Acetate kinase from *Methanosarcina thermophila*

SEQ ID 26 DNA sequence encoding the Adenylate kinase from *Sulfolobus acidocaldarius*

SEQ ID 27 DNA sequence encoding the Adenylate kinase from *Sulfolobus acidocaldarius*, wherein codon usage has been optimised for expression of the gene in *E-coli*.

SEQ ID 28 DNA sequence encoding the Adenylate kinase from *Thermotoga maritima*

SEQ ID 29 DNA sequence encoding the Adenylate kinase from, *Thermotoga maritima*, wherein codon usage has been optimised for expression of the gene in *E-coli*.

SEQ ID 30 DNA sequence encoding the Adenylate kinase from *Archaeoglobus fulgidus*, wherein codon usage has been optimised for expression of the gene in *E-coli*.

Example 1**Assay Protocol for *Sulfolobus acidocaldarius* AK**

- 5 100 microlitres of a formulation of 1mg/ml of adenylate kinase on a suitable solid support is the indicator and is subjected to the treatment process as required.

- 10 A heat inactivation step is optional. This involves heating the sample to a temperature permissive for the indicator AK but above that at which any mesophilic AKs are denatured. Typically this is by incubation at 80°C for 10 minutes.

- 15 A washing step is optional and may be incorporated to remove any trace of materials used to perform the treatment if they are found to interfere with the assay.

- 20 The indicator is then added to a tube containing ADP substrate (e.g. reagents from Celsis, Biothema, Promega) at a concentration of 13.5µM diluted in 15mM MgAc, 1mM EDTA buffer, pH 6.8 and incubated at 70°C for 20 minutes.

- 25 The sample is cooled to room temperature and luciferin/luciferase substrate (ATP Reagent, Thermo Life Science) added. The assay is incubated for the required length of time and a luminometer is used to read the sample.

Example 2**Purification of native adenylate kinase enzymes**

- 5 Biomass was produced from twenty-four diverse thermophilic and hyperthermophilic microorganisms (Table 1).

Eight members of the archaea were represented along with sixteen diverse aerobic and anaerobic bacteria. AKs from each of these organisms was
10 purified by affinity chromatography using selective absorption and desorption from Cibacron Blue 3A (Blue Sepharose). All enzymes were further characterised and purified by gel filtration (Superdex G200). This enabled identification of the major AK fraction and estimation of molecular mass.

15

20

25

30

Example 3

Analysis of thermostability of native adenylate kinases

- 5 The thermostability at 70, 80 and 90°C of adenylate kinases isolated from biomass from thermophilic organisms was assessed, and the results shown in Fig. 1.

10 The adenylate kinases were isolated from the biomass by affinity chromatography using selective absorption and desorption from Cibacron Blue 3A (Blue Sepharose). The samples eluted from the columns were diluted 1:10 000 and then 10µl of each added to a microtitre well. 2.5µl of apyrase was added to each well to destroy the ATP present from the elution buffer, and incubated at 37°C for 30 minutes. The apyrase was inactivated by heat
15 treatment at 65°C for 20 minutes.

ADP substrate was added and incubated at either 70 (panel A), 80 (panel B) or 90°C (panel C) for 30 minutes and cooled to 25°C before the addition of 10µl of D-luciferin-luciferase reagent. The ATP produced was measured as
20 RLU on a plate luminometer.

25

30

Example 4

Expression and purification of recombinant adenylate kinases

- 5 Clones expressing representative thermostable AKs were secured and recombinant thermostable AKs from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* and the thermophilic bacterium, *Bacillus stearothermophilus* produced. The plasmids were transformed into *E.coli* and the cell extracts shown to contain protein bands on electrophoresis
- 10 corresponding to the expected molecular masses of the AKs. Thermostable AK activity was measured after incubation at the appropriate temperature (80°C for the *Sulfolobus acidocaldarius* AK and 60°C for the *Bacillus stearothermophilus* AK).
- 15 Purification methods for both thermostable AKs were established and included an initial heat treatment of incubation for 20min at 80°C, to inactivate and aggregate proteins derived from *E.coli*, followed by affinity chromatography and gel filtration. The affinity chromatography involved adsorption of the enzyme to Blue Sepharose, followed by specific elution with a low
- 20 concentration of AK co-factors (AMP+ATP and magnesium ions). The ATP and AMP (Sigma) in the elution buffer were degraded by incubation with mesophile apyrase, which is readily inactivated by subsequent heat treatment. Gel filtration chromatography was scaled up to utilise a preparation grade Superdex column to enable large quantities of both enzymes to be prepared.
- 25 Primers were designed for PCR amplification of the AK genes from the thermophilic organisms identified during the screening of candidate native enzymes.
- 30 The thermostable microorganisms were grown using individually defined growth conditions and genomic DNA isolated and used as templates for PCR amplification of the adenylate kinase genes from each organism. PCR amplified adenylate kinase genes from the thermophilic organisms, *Thermotoga maritima*, *Aeropyrum pernix*, *Sulfolobus acidocaldarius* and

Sulfolobus solfataricus were sub-cloned into the vector, pET28a and transformed into a codon enhanced *E.coli* strain expressing rare tRNAs (Zdanovsky et al, 2000). This *E.coli* strain is suitable for enhancing expression levels of AT-rich genes.

5

The success of the transformation was assessed by a mini-expression study, and the results analysed by SDS-PAGE of the culture supernatants before and after induction with IPTG. SDS-PAGE was also used to analyse the supernatants after inclusion of a heat treatment step, which consisted of heating the sample to 80°C for 20 minutes prior to running on the SDS-PAGE gel to remove heat labile proteins present in the sample.

10

Sequences:

15 Seq IDs

1 - Adenylate kinase from *Sulfolobus solfataricus*

MKIGIVTGIP GVGKTTVLSF ADKILTEKGI SHKIVNYGDY MLNTALKEGY
20 VKSRDEIRKL QIEKQRELQA LAARRIVEDL SLLGDEGIGL IDTHAVIRTP
AGYLPGLPRH VIEVLSPKVI FLLEADPKII LERQKRDSSR ARTDYSDTAV
INEVIQFARY SAMASAVLVG ASVKVVVNQE GDPSIAASEI INSLM

2 - Adenylate kinase from *Sulfolobus acidocaldarius*

25

MKIGIVTGIP GVGKSTVLAK VKEILDNQGI NNKIINYGDF MLATALKLGY
AKDRDEMRLK SVEKQKKLQI DAAKGIAEEA RAGGEGYLF I DTHAVIRTPS
GYLPGLPSYV ITEINPSVIF LLEADPKIIL SRQKRDTRN RNDYSDESVI
LETINFARYA ATASAVLAGS TVKVIVNVEG DPSIAANEII RSMK

30

3 - Adenylate kinase from *Sulfolobus tokodaii*

MSKMKIGIVT GIPGVGKTTV LSKVKEILEE KKINNKIVNY GDYMLMTAMK
LGYVNNRDEM RKLPEKQKQ LQIEAARGIA NEAKEGGDGL LFIDTHAVIR

TPSGYLPGLP KYVIEEINPR VIFLLEADPK VILDRQKRDT SRSRSDYSDE
RIISETINFA RYAAMASAVL VGATVKIVIN VEGDPAVAAN EIINSML

4 - Adenylate kinase from *Pyrococcus furiosus*

5

MPFVVIITGI PGVGKSTITR LALQRTKAKF RLINFGDLMF EEAVKAGLVK
HRDEMRKLPL KIQRELQMKA AKKITEMAKE HPILVDTHAT IKTPHGYMLG
LPYEVVKTLN PNFIVIEAT PSEILGRRLR DLKRDRDVET EEQIQRHQDL
NRAAAIAYAM HSNALIKIIE NHEDKGLEEA VNELVKILD DL AVNEYA

10

5 - Adenylate kinase from *Pyrococcus horikoshii*

MPFVVIITGI PGVGKSTITK LALQRTRAKF KLINFGDLMF EEALKLKLVK
HRDEMRKLPL EVQRELQMNA AKKIAEMAKN YPILLDTHAT IKTPHGYLLG
15 LPYEVIKILN PNFIVIEAT PSEILGRRLR DLKRDRDVET EEQIQRHQDL
NRAAAITYAM HSNALIKIIE NHEDKGLEEA VNELVKILD DL AVKEYA

6 - Adenylate kinase from *Pyrococcus abyssi*

20 MSFVVIITGI PGVGKSTITR LALQRTKAKF KLINFGDLMF EEAVKAGLVN
HRDEMRKLPL EIQRDLQMKV AKKISEMARQ QPILLDTHAT IKTPHGYLLG
LPYEVIKTLN PNFIVIEAT PSEILGRRLR DLKRDRDVET EEQIQRHQDL
NRAAAIAYAM HSNALIKIIE NHEDKGLEEA VNELVEILD DL AVKEYA

25 7 - Adenylate kinase from *Methanococcus thermolithotrophicus*

MKNKLVVVTG VPGVGGTTIT QKAMEKLSEE GINYKMVNFG
TVMFEVAQEE NLVEDRDQMR KLDPDTQKRI QKLAGRKIAE
MVKESPVVVD THSTIKTPKG YLPGLPVWVL NELNPDIIIV VETSGDEILI
30 RRLNDETRNR DLETTAGIEE HQIMNRAAAM TYGVLTGATV KIIQNKNNLL
DYAVEELISV LR

8 - Adenylate kinase from *Methanococcus voltae*

MKNKVVVVTG VPGVGSTTSS QLAMDNLKE GVNYKMVSFG
SVMFEVAKEE NLVSDRDQMR KMDPETQKRI QKMAGRKIAE
MAKESPVAVD THSTVSTPKG YLPGLPSWVL NELNPDLIIV VETTGDEILM
RRMSDETRVR DLDTASTIEQ HQFMNRCAAM SYGVLTGATV
5 KIVQNRNGLL DQAVEELTNV LR

9 - Adenylate kinase from *Methanococcus jannaschii*

MMMMKNKVVV IVGVPGVGST TVTNKAIEEL KKEGIEYKIV NFGTVMFEIA
10 KEEGLVEHRD QLRKLPPEEQ KRIQKLAGKK IAEMAKEFNI VVDTHSTIKT
PKGYPGLPA WVLEELNPD IIVLVEAENDE ILMRRLKDET RQRDFESTED
IGEHI FMNRC AAMTYAVLTG ATVKIIKNRD FLLDKAVQEL IEVLK

10 - Adenylate kinase from *Methanopyrus kandleri*

15 MGYVIVATGV PGVGATTVTT EAVKELEGYE HVNYGDVMLE IAKEEGLVEH
RDEIRKLPAE KQREIQRLAA RRIAKMAEEK EGIIVDTHCT IKTPAGYLP
LPIWVLEELQ PDVIVLIEAD PDEIMMRRVK DSEERQRDYG RAHEIEEHQK
MNRMAAMAYA ALTGATVKII ENHDDRLEEA VREFVETVRS L

20

11 - Adenylate kinase from *Methanotorris igneus*

MKNKVVVVTG VPGVGGTTLT QKTIEKLKEE GIEYKMVNFG TVMFEVAKEE
GLVEDRDQMR KLD PDTQKRI QKLAGRKIAE MAKESNVIVD THSTVKTPKG
25 YLAGLPIWVL EELNPDIIVI VETSSDEILM RRLGDATRNR DIELTSIDE
HQFMNRCAAM AYGVL TGATV KIIKNRDGLL DKAVEELISV LK

12 - Adenylate kinase from *Pyrobaculum aerophilum*

30 MKIVIVALPG SGKTTILNFV KQKLPDVKIV NYGDVMLEIA KKRFGIQHRD
EMRKKIPVDE YRKVQEEAAE YIASLTGDVI IDTHASIKIG GGYYPGLPDR
IISKLPDVI LLEYDPKVI LERRKKDPDR FRDLESEEEI EMHQQANRY
AFAAANAGES TVHVLNFRGK PESRPFEHAE VAAEYIVNLI LRTRQKS

13 - Adenylate kinase from *Thermotoga maritima*

MMAYLVFLGP PGAGKGTYAK RIQECTGIPH ISTGDIFRDI VKKENDELGK
KIKEIMEKGE LVPDELVNEV VKRRLSEKDC EKGFIIDGYP RTVAQAEFLD
5 SFLESQNKQL TAAVLFDVPE DVVVQRLTSR RICPKCGRIY NMISLPPKED
ELCDDCKVKL VQRDDDKEET VRHRYKVYLE KTQPVIDYYG KKGILKRVDG
TIGIDNVVAE VLKIIGWSDK

14- Adenylate kinase from *Aeropyrum pernix*

10

MKVRHPFKVV VVTGVPGVGK TTVIKELQGL AEKEGVKLHI VNFGSFMLDT
AVKLGLVEDR DKIRTLPLRR QLELQREAAK RIVAEASKAL GGDGVLIIDT
HALVKTVAGY WPGLPKHVLD ELKPDMAVV EASPEEVAAR
QARDTTRYRV DIGGVEGVKR LMENARAASI ASAIQYASTV AIVENREGEA
15 AKAAEELLRL IKNL

15- Adenylate kinase from *Archaeoglobus fulgidus*

MNLIFLGPPG AGKGTQAKRV SEKYGIPQIS TGDMLREAVA KGTELGKKAK
20 EYMDKGELVP DEVVIGIVKE RLQQPDCEKG FILDGFPRTL AQAEALDEML
KELNKKIDAV INVVPPEEEV VKRITYRRTC RNCGAVYHLI YAPPKEDNKC
DKCGGELYQR DDKEETVRE RYRVYKQNTD PLIDYYRKKG ILYDVDGTDK
IEGVWKEIEA ILEKIKS

25 16- Monomeric adenylate kinase (AdkE) from *Pyrococcus abyssi*

MNIFLGPPG SGKSTQARRI TERYGLTYIA SGDIIRAEIK ARTPLGIEME
RYLSRGDLIP DTIVNTLIIS KLRRVRENFI MDGYPRTPAQ VITLENYLYD
HGIKLDVAID IYITKEESVR RISGRRICSK CGAVYHVEFN PPKVPGKCDI
30 CGGELIQRPD DRPEIVEKRY DIYSKNMEPI IKFYQKQGIY VRIDGHGSID
EVWERIRPLL DYIYNQENRR

Example 5**Analysis of the thermostability of recombinant adenylate kinases**

- 5 The thermal stability of recombinant tAK enzymes was assessed in crude *E.coli* cell lysates.

Cells were grown essentially as described in Example 4 and lysed by sonication. The AK activity of the crude extract was determined both before
10 and after heat treatment at 80°C for 30 minutes followed by 10-fold serial dilution

The results (see Figure 4) demonstrate that a wide variety of recombinant enzymes are suitable for the use in the method of the invention. Particularly
15 preferred AKs are those from *T.maritima*, *A.fulgidus* and *S.solfataricus*. Such enzymes are likely to provide a greater dynamic range for the bioluminescent assay, if required, to provide still further sensitivity.

20

25

30

Example 6

The properties of specific thermostable adenylate kinases demonstrate their value as indicators for TSE inactivation.

5

The prion molecule shows a remarkably high propensity to stick to steel surfaces.

10

To develop an indicator for validation of prion decontamination procedures, a kinase having similar properties to the prion molecule would be advantageous. To explore this concept, the binding of different recombinant thermostable adenylate kinases to a given surface was assessed (see Figure 5).

15

The binding of the kinases to previously blocked plates was assessed. The surface of a standard polystyrene microtitre plate was blocked by the addition of 5% skimmed milk. The milk was removed and dilutions of thermostable adenylate kinases from *Archaeoglobus fulgidus* (Afu), *Thermotoga maritime* (Tma) and *Sulfolobus acidocaldarius* (Sac) were applied to the plate. After washing, the amount of bound kinase was measured as described for the standard assay method (Example 1). The results demonstrate that the Sac kinase showed significantly higher binding to the blocked plate than either of the two alternative kinases. Whilst the specific activity of the enzymes was not identical, both Afu and Tma kinases showed higher activity than that from Sac, such that the real difference in binding is even more exaggerated than shown in the figure.

20

25

30

As skimmed milk is a commonly used blocking agent for reducing protein binding to plates, these results demonstrate that the Sac kinase has an extremely strong tendency for hydrophobic adsorption to the surface used in these experiments (polystyrene). This property of the enzyme, similar to that observed for the prion molecule to steel surfaces, means that it is an extremely effective indicator for assessing a wide range of TSE inactivation and/or removal processes. The Sac kinase binds in a very similar way to

stainless steel, and shows a co-operativity of binding (i.e. there is a logarithmic (rather than linear) relationship between bound activity and amount of enzyme at low concentrations suggesting that it has aggregative properties on this surface similar to those seen for prion molecules).

5

This feature of the enzyme demonstrates its potential for direct adsorbance type indicators where the kinase is adsorbed directly onto a suitable support rather than being covalently attached. The indicator in this case functions by assessing the ability of a process to remove the material from the surface and

10 is widely applicable to monitoring washing and decontamination processes.

15

20

25

30

Example 7**Genetic modification of adenylate kinases to improve stability**

- 5 Site-directed mutants were constructed in the AK gene from *P.furiosus*, *P.horikoshii* and *S.acidocaldarius* as shown in Examples 8-10 and SEQ IDs 17-19 respectively, using standard methods known to those familiar with the art.
- 10 In addition to specific changes identified in each gene, the regions underlined in the *S.acidocaldarius* sequence form the core packing region of the archaeal adenylate kinase trimer structure. Hence amino acid substitutions that disturb the packing of this region are likely to have a major effect in decreasing the thermal and physical stability of the enzyme. Conversely amino acid
- 15 substitutions that improve the core packing, in particular hydrophobic residues with large side chains, may stabilise the enzyme to heat or other processes. Therefore in addition to the specific mutations already described a number of “selective” approaches were used with localised gene shuffling of related gene sequences in these regions (essentially as described in Stemmer (1994)
- 20 Nature 370:389-391 and Crameri et al (1996) Nature Biotech. 14:315-319) and random PCR-based mutagenesis using degenerate oligonucleotides or modified nucleotide mixes (e.g. Vartanian et al (1996) Nucleic Acid Res.24:2627-2633). A number of these modifications show altered stability when assessed by recombinant expression in *E.coli* and rapid assay of
- 25 adenylate kinase activity in lysed cells at high temperature.

Example 8

Adenylate kinases from *Pyrococcus furiosus* genetically engineered to provide improved stability (SEQ ID NO. 17).

5

MPFVVIITGI PGVGKSTITR LALQRTKAKF RLINFGDLMF EEAVKAGLVK
HRDEMRKLPL (**K TO E**) IQRELQMKA AKKI (**T TO A**) EMAKE HPILVDTHAT
IKTPHGY (**M TO L**) LG LPYEVVKTLN PNFIVIEAT PSEILGRRLR
DLKRDRDVET EEQIQRHQDL NRAAAIAYAM HSNALIKIIE NHEDKGLEEA
10 VNELVKILDL AVNEYA

15

Mutations at one or more or all of the sites indicated modify the thermostability of the enzyme. In addition to the three defined changes highlighted, modification of the alanine at position 157 to another small hydrophobic residue (such as I, L) or larger hydrophobic residue (such as F) increases the thermostability of the recombinant protein. Hence, there are 35 variants possible through combination of modifications at these sites. Modification of amino acid 157 to a polar residue such as the T (as observed at the equivalent position in AdkA of *P.horikoshii*), S Y, D, E, K, R results in a decrease in stability.

20

25

30

Example 9

Adenylate kinases from *Pyrococcus horikoshii* genetically engineered to provide improved stability (SEQ ID NO. 18).

5

The modification of either or both of the residues shown in bold and underlined increases the thermal stability of the enzyme (3 variants are possible).

10 MPFVVIITGI PGVGKSTITK LALQRTRAKF KLINFGDLMF EEALKL**GL**VK
HRDEMRKLPL EVQRELQMNA AKKIAEMAKN YPILLDTHAT IKTPHGYLLG
LPYEVIKILN PNFIVIIIEAT PSEILGRRLR DLKRDRDVET EEQIQRHQDL
NRAAAI**A**YAM HSNALIKIIE NHEDKGLEEA VNELVKILD AVKEYA

15

20

25

30

Example 10

Adenylate kinase from *Sulfolobus acidocaldarius* genetically engineered to provide improved stability (SEQ ID NO. 19).

5

The modification of the underlined residues shown can increase the thermal stability of the enzyme.

10

MKIGIVTGIP GVGKSTVLAK VKEILDNQGI NNKIINYGDF MLATALKLG
AKDRDEMRL SVEKQKKLQI DAAKGIAEEA RAGGEGYLF DTHAVIRTPS
GY (A TO M) PGLPSYV ITEINPSVIF LLEADPKIIL SRQKRDTTRN
RNDYSDESVI LETINFARYA ATASAVLAGS TVKVIVNVEG DPSIAANEII
RSMK

15

20

25

30

Example 11

An adenylate kinase indicator for the validation of inactivation of TSE

- 5 AK-based prion indicators were developed for use with protease inactivation of TSE material at elevated temperature and pH.

Indicator 1

- 10 A polycarbonate support was coated with a formulation of purified recombinant *S.acidocaldarius* AK (as described in Example 4). The enzyme was formulated at a concentration of 1mg/ml in the presence of 5% (w/v) sorbitol, 10mg/ml bovine serum albumen (Fraction V; Sigma chemical company) in phosphate buffered saline pH 7.4 (PBS). A volume of 100µl was
15 dried onto the support at 22°C for 1 hour.

Indicator 2

- A polystyrene support was coated with 100 microlitres of a formulation
20 containing 1mg/ml *S.acidocaldarius* AK, 1mM tryptophan, 5% (w/v) sorbitol in Tris buffered saline (TBS) pH 7.4 and dried for 24 hours at 4 degrees C.

Indicator 3

- 25 A third indicator was prepared by cross-linking 100 microlitres of a 1mg/ml formulation of a thermostable adenylate kinase from *S.solfataricus* onto a flexible polystyrene wand using a method based on disulfide bond formation. The recombinant thermostable adenylate kinase was derivitised with the heterobifunctional agent Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-
30 propionamido)hexanoate (SPDP; Pierce chemical company, UK) at a ratio of between 1 and 3 SPDPs: protein. The derivatised kinase was then reduced by reaction with the reducing agent dithiothreitol (DTT), the reducing agent removed by dialysis, and the kinase reacted with a maleimide derivatised polystyrene wand.

Protease treatment

An instrument wash bath was set up to operate at 60°C and an alkaline protease formulation added to give between 1.5 and 2 mg/ml of enzyme buffered at pH 12 (as measured at the bath temperature). Suitable anionic detergents may also be included in the formulation if desired. The indicator was incubated for 30 minutes under the conditions described. The indicator was then removed and rinsed once with distilled water. The enzyme activity was then measured as described in Example 1 and luminescence measured using a luminometer.

Standard curves for adenylate kinase enzyme

Standard curves for the AK enzyme was prepared as follows. Serial dilutions of purified *Sulfolobus acidocaldarius* AK from 10 microgrammes/ml to 1fg/ml were prepared in 50mM Tris, 25mM MESNA, pH 7.3. 100 microlitres of enzyme was added to each well of a microtitre plate and 100 microlitres of 135micromolar ADP 15mM MgAc, 1mM EDTA added to each well. Three separate standard curves were prepared for incubation of the assay plate at 30 degrees C, 50 degrees C and 70 degrees C for 20 minutes. Following incubation 30 microlitres of luciferin/luciferase reagent (Biothema) was added and the signal read in a luminometer (Orion, Berthold) and the results shown in Figure 3.

Validation of TSE inactivation

The level of kinase activity was assessed against the standard curve for the kinase. For an indicator carrying 100 microgrammes of kinase with a luminescence value of greater than 1,000,000 Relative Light Units (RLU), as assessed from the standard curve, a luminescence value of less than 4000 RLU corresponds to a 6-log reduction in the concentration of kinase, and a luminescence value of less than 500 RLU corresponds to an 8-log reduction in the concentration of kinase.

Under the protease digestion conditions described above (2mg/ml alkaline protease designated MC3 in a buffer formulation at pH12 and digestion for 30 minutes at 60 degrees C) we demonstrated a reduction of approximately 8-logs in the levels of infectivity of the BSE-301V strain bioassayed in VM mice.

5 Under the same conditions an AK indicator formulated as described above showed a reduction in RLU from in excess of 1,000,000 (untreated) to less than 500 RLU. Thus, an 8 log reduction in the concentration of kinase corresponds to an 8-log reduction in infectivity of BSE. Using this fact, it is possible to monitor kinase concentration and relate it to the reduction in levels
10 of BSE infectivity.

Use of validated procedure

A set of surgical instruments used in routine surgery or routine neurosurgery
15 for a patient not known to be incubating any form of CJD is returned to a hospital sterile services unit following use. The instruments are prepared for routine cleaning in a washer/disinfector set to operate at 60°C for 30 minutes with a formulation of alkaline protease MC3. One or more AK indicators as described above are included in the processing bath. After treatment, and
20 before instruments are sent on for normal autoclave sterilisation, the indicator is removed and a rapid assay carried out to confirm that the process is effective. An assay result that demonstrates a 6- or 8-log loss of activity, as defined by the parameters of the process, is required before any instruments can be processed further. Following a successful process, instruments may
25 be prepared for other sterilisation procedures such as autoclaving as required.

Example 12**Expression of acetate and pyruvate kinases**

- 5 Following the methods of Example 4, we expressed acetate and pyruvate kinases:-

20 - Acetate kinase from *Thermatoga maritima*

10 MRVLVINS GS SSIKYQLIEM EGEKVLCKGI AERIGIEGSR LVHRVGDEKH
VIERELPDHE EALKLILNTL VDEKLGVIKD LKEIDAVGHR VVHGGERFKE
SVLVDEEV LK AIEEV SPLAP LHN PANLMGI KAAMKLLPGV PNVAVFD TAF
HQTIPQKAYL YAIPEYYEK YKIRRYGFHG TSHRYVSKRA AEILGKKLEE
LKIITCHIGN GASVA AVKYG KCVDTSMGFT PLEGLVMGTR SGD LDP AIPF
15 FIMEKEGIS P QEMYDILNKK SGVYGLSKGF SSDMRDIEEA ALKGDEWCKL
VLEIYDYRIA KYIGAYAAAM NGVDAIVFTA GVGENSEPITR EDVCSYLEFL
GVKLDKQKNE ETIRGKEGII STPDSRVKVL VVPTNEELMI ARDTKEIVEK
IGR

20 21 - Pyruvate kinase from *Pyrococcus horikoshii*

MRRMKLP SHK TKIVATIGPA TNSKKMIKKL IEAGMNVARI NFSHGTFEEH
AKIEMVREQ SQKLDRRVAI LADLPGLKIR VGEIKGGYVE LERGEKVTLT
TKDIEGDETT IPVEYKDFPK LVSKGDVIYL SDGYIVLRVE DVKENEVEAV
25 VISGGKLF SR KGINIPKAYL PVEAITPRDI EIMKFAIEHG VDAIGLSFVG
NVYDVLKAKS FLERNAGAGDT FVIKIERPD AVRNFNEILN AADGIMIARG
DLGVEMPIEQ LPILQKRLIR KANMEGKPVI TATQMLVSMT MEKVPTRAEV
TDVANAILDG TDAVMLSEET AVGKFPIEAV EMMARIAKVT EYRESFGIT
RMREFLEGTK RGTIKEAITR SIIDAICTIG IKFILTPTKT GRTARLISRF
30 KPKQWILAFS TREKVCNNLM FSYGVYPFCM EEGFNENDIV RLIKGLGLVG
SDDIVLMTEG KPIEKT VGTN SIKIFQIA

22 - Pyruvate kinase from *Sulfolobus solfataricus*

MRKTKIVATL GPSSEEKVKE LAEYVDVFRI NFAHGDETSR RKYFDLIRTY
APESSIIVDL PGPKLRLGEL KEPIEVKKGD KIVFSQKDG I PVDDSELFYSA
VKENSDILIA DGTIRVRVKS KAKDRVEGTV IEGGILLSRK GINIPNVNLK
SGITDNDLKL LKRALDLGAD YIGLSFVISE NDVKKVKEFV GDEAWVIAKI
5 EKSEALKNLT NIVNESDGIM VARGDLGVET GLENLPLIQR RIVRTSRVFG
KPVLATQVL TSMINSPIPT RAEIIDISNS IMQGVDSIML SDETAIGNYP
VESVRTLHNI ISNVEKSVKH RPIGPLNSES DAIALAAVNA SKVSKADVIV
VYSRSGNSIL RVSRLRPERN IIGVSPDPRL AKKFKLCYGV IPISINKKMQ
SIDEIIDVSA KLMQEIKDL KFKKIVIVGG DPKQEAGKTN FVIVKTLEQQ KK

10

23 - Pyruvate kinase from *Thermotoga maritima*

MRSTKIVCTV GPRTDSYEMI EKMIDLG VNV FRINTSHGDW NEQEQKILKI
15 KDLREKKKKP VAILIDLAGP KIRTGYLEKE FVELKEGQIF TLTTKEILGN
EHIVSVNLSS LPKDVKKGDT ILLSDGEIVL EVIETTDTEV KTVVKVGGKI
THRRGVNVPT ADLSVESITD RDREFIKLGT LHDVEFFALS FVRKPEDVLK
AKEEIRKHGK EIPVISKIET KKALERLEEI IKVSDGIMVA RGD LGVEIPI
EEVPIVQKEI IKLSKYYSKP VIVATQILES MIENPFPTRA EVTDIANAIF
20 DGADALLTA ETAVGKHPL EAIKVL SKVAK EAEKKLEFFR TIEYDTSDIS
EAISHACWQL SESLNAKLII TPTISGSTAV RVSKYNVSQP IVALTPEEKT
YYRLSLVRKV IPVLAEKCSQ ELEFIEKGLK KVEEMGLAEK GDLVVLTSKV
PGKVGTTNTI RVLKVD

25

24 - Pyruvate kinase from *Pyrococcus furiosus*

MRRVKLP SHK TKIVATIGPA TNSRKMIKQL IKAGMNVARI NFSHGSFEEH
30 ARVIEIREE AQKLDRRVAI LADLPGLKIR VGEIKGGYVE LKRGEKVILT
TKDVEGDETT IPVDYKGFNP LVSKGDIIYL NDGYIVLKVE NVRENEVEAV
VLSSGGKLF SR KGVNIPKAYL PVEAITPKDF EIMKFAIEHG VDAIGLSFVG
SVYDVLKAKS FLEKNNAEDV FVIAKIERPD AVRNFDEILN AADGIMIARG
DLGVEMPIEQ LPILQKKLIR KANMEGKPIV TATQMLVSMT TEKVPTRAEV
35 TDVANAILDG TDAVMLSEET AIGKFPIETV EMMGKIAKVT EEYRESFGLS
RIREFMEIKK GTIKEAITRS IIDAICTIDI KFIPTPTRTG RTARLISRFK

PKQWILAFST NERVCNNLMF SYGVYPFCLE EGFDENDIVR LIKGLGLVES
DDMVLMTGK PIEKTVGTNS IKIFQIA

25 - Acetate kinase from *Methanosarcina thermophila*

5

MKVLVINAGS SSLKYQLIDM TNESALAVGL CERIGIDNSI ITQKKFDGKK
LEKLTDLPTH KDALEEVVKA LTDDEFGVIK DMGEINAVGH RVVHGGEKFT
TSALYDEGVE KAIKDCFELA PLHNPPNMMG ISACAEIMPG TPMVIVFDTA
FHQTMPPYAY MYALPYDLYE KHGVRKYGFH GTSHKYVAER

10

AALMLGKPAE ETKIITCHLG NGSSITAVEG GKSVETSMGF TPLEGLAMGT
RCGSIDPAIV PFLMEKEGLT TREIDTLMNK KSGVLGVSGL SNDFRDLDEA
ASKGNRKAEL ALEIFAYKVK KFIGEYS AVL NGADAVVFTA GIGENSASIR
KRILTGLDGI GIKIDDEKNK IRGQEIDIST PDAKVRVFVI PTNEELAIAR
ETKEIVETEV KLRSSIPV

15

20

25

30

Example 13**Thermostable adenylate kinase as an indicator for proteolytic inactivation of TSE agent.**

5

A 20mg/ml stock of alkaline protease was diluted using a solution of 10µg/ml recombinant *S.acidocaldarius* AK in buffered 0.2M KCl pH 12, to generate a range of protease concentrations from 2 mg/ml down to 0.001µg/ml. A volume of 100µl of material was added to a polystyrene thermocycler plate and incubated for 10 minutes at 60°C to allow digestion of the AK by the alkaline protease. The solution was neutralised by addition of 10µl of 10 x Phosphate buffer pH 7. 100µl/well of 135µM ADP in 15mM MgAc, 1mM EDTA buffer was added. The wells were then incubated for 20mins at 70°C in a thermocycler. 30µl/well of Luciferin/Luciferase (ATP) reagent (Biothema) was added and the wells read on the luminometer immediately. The results of the assay are shown in Figure 2.

These results show the remarkable stability of the AK enzyme to digestion by alkaline protease under conditions identified as being useful for the decontamination of surgical instruments. To relate the reduction observed following digestion with alkaline protease to the levels of activity remaining after treatment a standard curve such as that shown in Figure 3 is used. In turn this is related to the degree of reduction in the levels of infectivity using a titration curve of TSE. For use as an indicator, the assay is designed such that the standard curve for enzyme activity is clearly marked with a limit/threshold value that corresponds to an acceptable level of decontamination by the process.

30

Example 14

Validation of the performance of cloth washing cycles using biological detergents

5

Preparation of Indicator 1

A first indicator is prepared by cross-linking a thermostable adenylate kinase from *S.solfataricus* onto a flexible polystyrene wand using a method based on disulfide bond formation. In this method, the thermostable adenylate kinase is derivitised with a heterobifunctional agent such as Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (SPDP; Pierce chemical company, UK) at a ratio of between 1-3 SPDP:protein. The derivatised kinase is then reduced by reaction with a reducing agent such as dithiothreitol (DTT), or 2-mercaptoethanesulfonic acid (MESNA), the reducing agent removed by dialysis, and the kinase reacted with a maleimide-derivatised polystyrene surface. Typically, 0.1 mg of kinase is present on the indicator.

Preparation of Indicator 2

20

A second indicator is prepared by the non-specific adherence of a thermostable adenylate kinase from *S.acidocaldarius* onto a high-protein binding polystyrene strip. The kinase is prepared at a concentration of 0.5-2 mg/ml in a bicarbonate buffer (pH 9.6), optionally containing the stabilising agent sorbitol at between 0.1 and 2% w/v. The kinase in binding buffer is then incubated with the high protein-binding polystyrene strip for a period of 1-2 hours at 22°C (or 4°C overnight). The residual kinase is removed by washing in a phosphate buffered saline. Typically, 0.1 mg of kinase is present on the indicator.

30

Validation of wash cycles

The washer is loaded with the items to be washed, and the indicator is fixed within a suitable holder on the inside of a washer (to facilitate its recovery). The wash cycle is then performed. At completion of the cycle, the indicator is removed and the residual activity of the kinase is assessed using a "reader" that is based on the assay methods described in Example 1. This reader is calibrated to indicate an acceptable "threshold" level of residual kinase activity within the indicator. This "threshold" level is derived from previous calibration and assessment of suitable performance within the process.

- 10 If the reader indicates that the residual kinase activity is equal to or below this threshold level, then the load is cleared for further processing.

15

20

25

30

Example 15

Validation of a process for the inactivation of Norwalk-like virus (also termed Norovirus, Norwalk virus or Winter Vomiting Disease) on hard surfaces using a protease formulation

Preparation of the Indicator

An indicator is prepared by immobilising 0.1mg of a thermostable adenylyate kinase from *S.acidocaldarius* on a thin polystyrene strip using the disulfide mediated cross-linking as described in Example 14.

Validation

The indicator strip is placed on the potentially contaminated surface. Both the surface and the indicator strip are then coated with a highly active alkaline protease, proteinase K, that has been formulated within a paste. (Formulating the proteinase K in a paste improves contact between the enzyme with the surface/indicator.) The paste is left on the surface/indicator for 1-2 hours.

At the end of the treatment, the indicator is removed and the level of residual kinase activity is assessed using the "reader" described in Example 14. If the residual kinase activity is equal to or below the "threshold" level of the reader, then the surface is cleared as safe for use.

Example 16**Validation of the protease degradation of ricin****5 Preparation of Indicator**

An indicator is prepared by immobilising 0.1mg of a thermostable acetate kinase from *Thermotoga maritima* onto a polycarbonate strip using the standard sulfhydryl-maleimide coupling method described in the text and in

10 Example 14.

Validation

The indicator strip is either immersed in a protease solution along with the
15 object to be decontaminated, as in Example 11, or is attached to the surface to be decontaminated, as in Example 15.

The object/surface is then treated with the protease solution for the required length of time. At the end of the treatment, the indicator is removed and the
20 level of residual kinase activity is assessed using a "reader" as described in Example 14. If the residual kinase activity is equal to or below the "threshold" level of the reader, then the object/surface is cleared as safe for use.

25

30

Example 17**Validation of the gas phase ozone inactivation of a TSE agent****5 Preparation of Indicator 1**

A first indicator is prepared by covalently cross-linking 0.1mg of a thermostable adenylate kinase from *S.acidocaldarius* onto a rigid polyvinyl chloride (PVC) support using the SPDP-maleimide method as described in
10 Example 14.

Preparation of Indicator 2

A second indicator is prepared by reacting the *S.acidocaldarius* adenylate
15 kinase with Bis-[β -(4-Azidosalicylamido)ethyl]disulfide (BASED; Pierce chemicals) in the presence of an amine reactive PVC surface. This leads to rapid covalent attachment of the kinase to the surface via amide linkages.

Validation

20

The indicator and the TSE-contaminated article are exposed to the gas phase ozone inactivation process. At the end of the process, the indicator is removed and the residual level of kinase activity is assessed using a "reader" as described in Example 14. If the residual kinase activity is equal to or below
25 the "threshold" level of the reader, then the article is cleared as safe for use.

30

Example 18**Validation of liquid chemical sterilisation systems (e.g. Endoclens) for cleaning endoscopes**

5

Preparation of Indicator 1

An indicator is prepared by chemically cross-linking (using the SPDP-based method described in Example 14) 0.1 mg of a thermostable adenylate kinase from *A.fulgidus* to the internal surface of a tube of a similar overall internal diameter to the endoscope tube.

Preparation of Indicator 2

15 A second indicator is prepared without derivatisation of the adenylate kinase. The gene sequence of the adenylate kinase from *Pyrococcus furiosus* is modified such that the recombinant protein includes an additional cysteine at the amino terminus of the sequence as shown in SEQ ID 4. The recombinant protein is expressed and purified as described previously and is reduced with
20 DTT to ensure that the sulfhydryl group is available. This can then be reacted directly with maleimide reactive plastic to generate the cross-linking required. The formulation of the cysteine-containing enzyme at around 1-2 mg/ml is similar to that required for the derivitised method described in Example 14.

25 Validation

The indicator apparatus is connected in series to the endoscope on the automatic reprocessing apparatus and the endoscope is then processed in the normal way. At the end of the process, the indicator is detached and
30 assessed for the level of residual kinase activity using a "reader" as described in Example 14. If the residual kinase activity is equal to or below the "threshold" level of the reader, then the endoscope is cleared as safe for use.

Example 19

Validation of cleaning of surgical instruments in hospital washer-disinfectors

5

Preparation of Indicator 1

An indicator is prepared by non-covalently attaching 0.1 mg of a thermostable adenylate kinase from *S.acidocaldarius* to a stainless steel surface. In this
10 example the protein is not covalently linked to the indicator device as the indicator is designed to measure the removal of surface soiling by the process. The kinase is formulated at between 1-2mg/ml preferably in a bicarbonate buffer at around pH 9.6. The material is adsorbed onto the metal
15 surface by incubation at room temperature for 1-2 hours. The metal surface may be modified to make it more hydrophobic if required (e.g. for high stringency washes additional attachment may be needed to ensure sufficient signal is retained on the indicator). Any unattached kinase is removed by washing the indicator under conditions that will not displace a significant proportion of the adhered indicator kinase.

20

Preparation of Indicator 2

A second indicator is prepared by adhering (using the methods described for Indicator 1) 0.1mg of the adenylate kinase from *S.acidocaldarius* to a stainless
25 steel surface as part of a formulation containing standard soils currently used for validating cleaning processes of this type. This enables the indicator technology to be correlated with methods acceptable to regulatory bodies as part of routine validation and maintenance of washer-disinfectors.

30 Validation

The indicator is included within the batch of surgical instruments to be processed, and the washer-disinfector cycle is run. At the end of the run, the indicator is removed and assessed for the level of residual kinase activity

using a “reader” as described in Example 14. If the residual kinase activity is equal to or below the “threshold” level of the reader, then the instruments are cleared as safe for use.

5

10

15

20

25

30

Example 20

Validation of processes for sterilising bulk liquids

5 Preparation of Indicator 1

A first indicator is prepared by covalently attaching 0.1mg of pyruvate kinase from *Sulfolobus solfataricus* to a polystyrene strip.

10 Preparation of Indicator 2

A second indicator is prepared by attaching 0.1mg of the thermostable adenylate kinase from *A.fulgidus* to the inner face of a semi-permeable membrane such as a dialysis tube. The *A.fulgidus* kinase contains a naturally occurring reactive cysteine residue (i.e. not disulfide-bonded within the native enzyme), which can be reacted with BMPH (Pierce). This generates a group capable of reacting with oxidised carbohydrates, as generated, for example, by the treatment of Visking tubing with a suitable oxidising agent. The enzyme is formulated as described in Example 14 and reacted with the oxidised membrane surface to generate a covalently linked indicator.

Validation

The indicator is then attached within the bulk liquid and the sterilisation process (such as autoclaving, the passage of oxidative gases or other chemical sterilisation) is carried out.

The indicator is removed from the bulk liquid on completion of the process, and the residual activity of the kinase is compared to a defined threshold as described in Example 14.

Example 21**Calibration of residual adenylate kinase enzyme activity following protease digestion.**

5

An illustration of a calibration curve for the assessment of suitable levels of inactivation of a prion agent is shown in Figure 6.

10

The example shown is for the protease treatment of surgical instruments under conditions defined as pH 12, 30°C for 30 minutes.

15

The protease concentration is defined by assessing the level of prion inactivation at different concentrations of protease under the conditions described (Panel A). In the illustration, the protease MC3 is capable of providing a level of inactivation equivalent to a 6-log reduction in infectious dose at a concentration of 1mg/ml under the conditions defined. At an equivalent concentration of the protease, and under the same conditions, the residual activity of adenylate kinase left on a *S.acidocaldarius* indicator, prepared essentially as described in Example 11, is of the order of 10RLU (Panel B). A value of less than 10 RLU is therefore required to allow a batch of instruments to be passed for re-use.

20

Optionally an additional safety margin is built in such that an RLU value of less than 1 is required, correlating to the level of residual adenylate kinase activity following treatment of the indicator with a concentration of protease that gives a 7-log reduction (1-log greater inactivation than the set standard) in prion infectivity.

25

30

Example 22**Calibration of residual adenylate kinase enzyme activity following ozone treatment**

5

An illustration of a calibration curve for the assessment of suitable levels of inactivation of a prion agent by an ozone treatment method is shown in Figure 7.

10 The example shown is for an ozone treatment that releases a fixed rate of ozone into the sterilisation chamber over a defined period. Levels of ozone are then calculated by increasing or decreasing the time, with a concomitant increase or decrease in ozone concentration.

15 In panel A, the levels of prion inactivation according to fractions or multiples of the standard cycle time are shown. 2.5 standard cycles therefore represents a 6-log inactivation of the prion. At an equivalent multiple of the ozone cycle the residual level of adenylate kinase left on an indicator, prepared essentially as described in Example 17, is of the order of 1000RLU.

20

A value of less than 1000 RLU is therefore required to allow a batch of instruments to be passed for re-use. Optionally an additional safety margin is built in for the indicator such that an RLU value of less than 100 is required, corresponding to the level of residual adenylate kinase activity following
25 treatment of the indicator with concentration of ozone that gives a 7-log reduction (1-log greater inactivation than the set standard) in prion infectivity.

30

Example 23**Calibration for an indicator designed to monitor routine cleaning of surgical instruments in hospital washer-disinfectors.**

5

Figure 8 shows an illustration of a calibration curve for assessing routine washing of surgical instruments using either a biological (enzyme containing) or detergent based formulation.

10 The indicator device is prepared as described in Example 19.

Panel A shows the percentage removal of standard soil from a defined medical instrument by the washer disinfectors run with a defined washer formulation. The times of the wash cycle are modified to identify the appropriate level of performance. Panel B shows the residual *S.acidocaldarius* kinase activity from an indicator device prepared as in Example 17 and washed under the same conditions as for the standardly soiled instrument.

The residual level of the soil is defined as 0.1% of the starting material within this test and this correlates with a wash time of 25 minutes. The RLU value for the indicator washed for 25 minutes is approximately 100 RLU. The indicator may therefore be used within a 30 minute wash cycle to provide an indication of acceptable "soil" removal at the 0.1% level with an RLU threshold of 100 or at a 0.05% level with an RLU cut-off of approximately 25.

25

30

Example 24**Modification of a hand held hygiene monitor to allow rapid read-out assessment of the kinase indicators.**

5

Many assay formats are potentially available to assess the levels of kinase activity associated with the indicators of the invention. These include tube luminometers, microtitre plate luminometers and a variety of other formats.

10 One format that has particular utility for the assessment of kinase activity on the indicator of the invention is the hand-held hygiene monitor. The current technology detects ATP, via a luciferin-luciferase enzyme system, either directly on a surface or by lysing bacteria or other cells removed from the surface.

15

Those familiar with the art will recognise that this system is amenable to adaptation for the detection of kinases on indicator devices as the system already contains the ability to produce and measure light in response to the presence of ATP. By changing the reagent formulation to add an ADP
20 susbtrate for the kinase it is possible to rapidly measure the presence of the enzyme on the indicator.

In practice, the indicator is added to a batch of material and treated. The indicator is then removed and inserted into a reagent tube to allow the
25 generation of ATP. The formulation of this reagent tube is essentially as described in the method for Example 1 with high purity ADP reagents. The tube is incubated for a defined length of time, as defined for the type of indicator and process being monitored. At the end of the incubation, the reagent sample containing the ATP generated by the presence of the residual
30 tAK is released into a second compartment containing the luciferin-luciferase, and measurable light is generated.

This device differs from the standard hygiene monitor as the first ATP-generation step is not required for the detection of ATP. The simplest way of

constructing such a device is to have a tube with 2 compartments separated by a breakable septum as illustrated in Figure 9. The indicator inserted into the tube at the first position (step 1) allows ATP generation and at the end of the incubation is pushed, either automatically or by hand, to the position shown in step 2 where light generation takes place. A wide variety of alternative constructions of such a device are possible. Optionally, heat denaturation steps to remove endogenous kinase activity and/or apyrase treatments to remove endogenous ATP can be carried out. The ATP generation step can be carried out at an elevated temperature if required.

10

15

| | <u>Organism</u> | <u>Domain</u> | <u>Growth</u> | <u>T_{opt}</u> | <u>pH_{opt}</u> |
|----|--|------------------|---------------------|------------------------|-------------------------|
| 1 | Aeropyrum pernix | Archaeon | Aerobe | 95°C | 7.0 |
| 2 | Alicyclobacillus acidocaldarius | Bacterium | Aerobe | 65°C | 3.5 |
| 3 | Aquifex pyrophilus | Bacterium | Microaerophi | 85°C | 6.5 |
| 5 | | | leerophile | | |
| 4 | Bacillus caldotenax BT1 | Bacterium | Aerobe | 65°C | 7.0 |
| 5 | Bacillus species PS3 | Bacterium | Aerobe | 65°C | 7.0 |
| 6 | Bacillus stearothermophilus 11057 | Bacterium | Aerobe | 65°C | 7.0 |
| 7 | Bacillus stearothermophilus 12001 | Bacterium | Aerobe | 65°C | 7.0 |
| 10 | 8 Bacillus thermocatenulatus | Bacterium | Aerobe | 65°C | 7.0 |
| 9 | Clostridium stercorarium | Bacterium | Anaerobe | 55°C | 7.0 |
| 10 | Meiothermus ruber | Bacterium | Aerobe | 60°C | 6.5 |
| 11 | Pyrococcus furiosus | Archaeon | Anaerobe | 95°C | 7.5 |
| 12 | Pyrococcus horikoshii | Archaeon | Anaerobe | 95°C | 7.0 |
| 15 | 13 Pyrococcus woesei | Archaeon | Anaerobe | 95°C | 7.0 |
| 14 | Rhodothermus marinus | Bacterium | Aerobe | 70°C | 6.5 |
| 15 | Sulfolobus acidocaldarius 98-3 | Archaeon | Aerobe | 75°C | 2.5 |
| 16 | Sulfolobus shibatae B21 | Archaeon | Aerobe | 75°C | 2.5 |
| 17 | Sulfolobus solfataricus P2 | Archaeon | Aerobe | 75°C | 2.5 |
| 20 | 18 Thermoanaerobacter ethanolicus | Bacterium | Anaerobe | 65°C | 6.0 |
| 19 | Thermoanaerobacter | Bacterium | Anaerobe | 65°C | 6.5 |
| | thermosulfurogenes | | | | |
| 20 | Thermobrachium celere | Bacterium | Anaerobe | 60°C | 7.0 |
| 21 | Thermococcus litoralis | Archaeon | Anaerobe | 85°C | 6.5 |
| 25 | 22 Thermus aquaticus YT1 | Bacterium | Aerobe | 70°C | 8.0 |
| 23 | Thermus caldophilus GK24 | Bacterium | Aerobe | 70°C | 8.0 |
| 24 | Thermus thermophilus HB8 | Bacterium | Aerobe | 70°C | 8.0 |

30 **Table 1**

List of thermophilic organisms cultured to produce biomass for isolation of thermostable AKs.